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Characterization of type 1 interferon production during persistent lymphocytic choriomeningitis virus (LCMV) infection

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ABSTRACT

Type 1 interferons (IFN-α/βs), are innate cytokines possessing important immunomodulatory and effector roles in the innate and adaptive response. They are transiently induced to very high levels during the initial stages of many viral infections. However, regulation of IFN-α/β production during persistent viral infections is not well understood. This project aimed to address these issues using the murine lymphocytic choriomeningitis virus (LCMV) infection model.

Initial objectives were to develop assays that would allow characterisation of the IFN-α/β response during chronic LCMV infection *in vivo* and *in vitro*. Cell-based reporter gene assays were developed to measure IFN-α/β activity, along with a series of real-time quantitative reverse-transcriptase polymerase chain reaction assays to detect mRNA transcripts of IFN-α/β, and their utility was evaluated.

Analysis of IFN-α/β production during acute and chronic LCMV infection indicated that there was an early burst of IFN production following infection, but only a low level of IFN-α/β production could be detected during the chronic phase of LCMV infection despite ongoing viral replication. Persistently-infected mice exhibited reduced numbers of splenic $CD11c^+$ plasmacytoid dendritic cells (DC). These animals were able to produce IFN-α/β in response toll-like-receptor (TLR) stimuli. Inoculation of influenza virus elicited less IFN-α/β production than was observed in uninfected mice, but this may have been due to impairment of influenza virus replication in persistently-infected mice.

In vitro LCMV infection of DC and fibroblast cell lines induced very little IFN-α/β production. Persistently-infected animals made normal IFN-α/β responses to TLR ligands but produced lower levels of IFN-α/β upon infection with Sendai virus than uninfected cells.

There are several pathways by which IFN-α/β can be induced. These results suggest that the intracellular response to RNA viruses may be impaired in LCMV-infected cells, although persistently-infected cells still retain some capacity to respond to external TLR stimuli.

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Glossary of abbreviations used in the text

µl	microlitres
µm	microgram
2'5' OAS	2,5' oligoadenylate synthetase
AP-1	Activator protein 1
APC	Allophycocyanin
APCs	Antigen presenting cells
BHK	Baby hamster kidney
BM-DC	Bone marrow derived dendritic cells
BVDV	Bovine diarrhoea virus
C	Cytoseine
CARD	N-terminal caspase recruiting domian
CARDIF	CARD adaptor inducing IFN-β
CAT	Chloramphenicol acetyl transferase
CBA	Cytokine Bead Array
cds	Coding sequence
CNS	Central nervous system
cpm	Counts per minute
Ct	Threshold cycle
CTL	Cytotoxic T lymphocyte
Cy5	Cytochrome 5
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbant assay
EMCV	Encephelomyocarditis virus
FACS	Fluorescence activated cell sorter
FAM	6-carboxyfluorescein
FITC	Fluorescein isothiocyanate
Flu	Influenza PR8
G	Guanine
GAS	γ activated sequence
GFP	Green fluorescent proterin
GM-CSF	Granulocyte-macrophage colony stimulating factor
GP	LCMV surface glycoprotein
GP-C	Glycooprotein precursor
HA	Haemagglutinin
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPRT	Hypoxanthine ribosyl-transferase
HPV	Human papilloma virus
hsp	Heat shock protein
HSV	Herpes simplex virus
i.c	Intra-cranial
i.p.	Intra-peritoneal
i.v.	Intra-venous
ICSP	IFN consensus sequence binding protein
IFN	Interferon
IFN-R	Type 1 IFN receptor

IFN-R	Type 1 IFN receptor
IFN-α	Interferon alpha
IFN-α/β	Type 1 interferons
IFN-β	Interferon beta
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IGR	Intergenic region
IL	Interleukin
IPS-1	IFN-β-promoter-stimulator
IRF	Interferon transcription factor
ISG	Interferon stimulated gene
ISRE	IFN-stimulated response element
JAK	Janus kinase
k/o	Knock-out
LCMV	Lymphocytic choriomeningitis virus
LCMV Arm	Lymphocytic choriomeningitis virus Armstrong strain
LCMV Cl13	Lymphocytic choriomeningitis virus Armstrong Clone1 13 isolate
LCMV Docile	Lymphocytic choriomeningitis virus Docile strain
LPS	Lipopolysaccharide
Luc	luciferase
M	mole
m.o.i.	Multiplicity of infection
mAb	Monoclonal antibody
MAP	Mitogen activated protein
MAVS	Mitochondrial antiviral signalling molecule
MCMV	Murine cytomegalovirus
MDA5	Melanoma differentiation associated gene 5
MEM	Modified Eagles medium
MGB	Major groove binder
MHC	Multiple histocompatability complex
min(s)	Minute(s)
ml	millilitres
mM	Micromole
MyD88	Myeloid differentiation marker 88
N.U	Neutralizing units
NDV	Newcastle disease virus
NF-κβ	Nuclear factor κβ
NK cells	Natural killer cells
NKT	Natural killer T-cells
NOD	Nucleotide oligomerization domain
NP	Nucleoprotein
nt	nucleotide
p.f.u.	Plaque forming units
PCR	Polymerase chain reaction
PD-1	Pregrammed death-1 receptor
PE	Phycoerythrin
PML	Promyelocytic leukaemia protein
PolyIC	Polyinosinic acid: Polycytidylic acid
PRD	Positive regulatory domain
PRR	Pattern recognition receptor
R848	Resiquimod
RIG-I	Retinoic acid inducible gene I
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute

RPMI	Roswell Park Memorial Institute
RT qPCR	One step revese transcriptase quantitative polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
SOCS	Suppressor of cytokine signalling
SPF	Specified pathogen free
ssRNA	Single-stranded ribonucleic acid
STAT	Signal transducer and activator of transcription
TAK	TGF-β activated kinase
TAMRA	6-carboxy-N,N,N',N',-tetramethylrhodamine
TANK	TRAF-associated NF-κβ activator
TBK-1	TANK binding kinase-1
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
T_H	T helper
TIR	TLR/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRIF/TICAM-1	TIR-domain containing adaptor-inducing IFN
U	Units of biological activity
UV	Ultraviolet
VRE	Viral recognition element
VSV	Vesicular stomatitis virus
VZV	Varicella zoster virus

CHAPTER 1

INTRODUCTION

1.1 Overview of immune activation in response to infection

Humans and other vertebrates are exposed from birth to a sustained barrage of organisms that pose a continuous threat to their well being. In mammals, evolution has given rise to two types of immunity, innate and acquired, that act in concert to protect the host from both external dangers in the form of invading pathogens, as well as internal threats in the form of aberrant host cells that may go on to become malignant cancers. Protection from cancer or infection by a pathogen is a multi-step process that depends upon interactions between multiple cell types of the immune system which need to occur in the proper sequence of events, location and temporal order so as to clear or control the infection with minimal damage to the host.

All vertebrates posses anatomical defences such as the carapace, epidermis of the skin or the mucus membranes of the gastro-intestinal lining that serve as a physical barrier. When an infectious agent successfully penetrates these defences and gains entry into the tissues or bloodstream, its presence is first detected by sentinel cells of the innate immune system. The innate arm, which includes neutrophils, eosinophils, macrophages, complement, fibronectin, dendritic cells (DC), natural killer (NK) cells, natural killer T cells (NKT) and mast cells is activated to mount a "non-specific" response that combats the infection and triggers the induction of an adaptive immune response, which may be required to eliminate the infection altogether. Cells of the innate immune system, such as dendritic cells, macrophages, monocytes, neutrophils and NK cells, do not require contact with specific antigens in order to be activated (Janeway and Medzhitov 2002). Instead, pattern recognition receptors e.g. the Toll-

like receptors (TLR) (Vasselon and Detners 2002), the mannose receptor (Milone and Fitzgerald-Bocarsly 1998) lectin receptors and cytoplasmic helicases such as retinoic-acid-inducible protein I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) recognise specific molecular patterns common to large groups of infectious agents and distinguishable from self. Slightly different responses are mounted according to the receptors triggered (Janeway and Medzhitov 2002), and the responses are usually optimized to eliminate the type of pathogen encountered. Innate activation, maturation and defence occur within minutes of detection and last for up to 72 hours, or sometimes more. The innate cells do not usually retain specific "memory" afterwards. The innate immune system therefore either clears the infection or controls it until more specific, potent and long lasting responses can be mounted by the adaptive or acquired immune response.

Unlike the innate immune response, the adaptive response is antigen-specific, and develops by clonal selection of CD8+ T-, CD4+ T- and B-lymphocytes from a vast repertoire of lymphocytes bearing antigen-specific receptors that are generated via gene rearrangement. Acquired immunity is responsible for the elimination of pathogens in the late phase of infection as well as the generation of immunological memory. However, the innate immune response is able to trigger and modulate the activation of the adaptive immune response. Thus, both arms of the immune system must be able to interact with each other in order to develop effective and long lived immunity against a given pathogen.

1.2 Innate and adaptive responses triggered by viral pathogens

Vertebrates are susceptible to infections by viruses, bacteria, fungi, prokaryotic and eukaryotic parasites and helminths and are able to tailor an immune response that is

appropriate to the type of pathogen encountered. As this project investigated aspects of the host response to lymphocytic choriomeningitis virus (LCMV), the primary focus of this review will be the immune response to viral pathogens.

Infection by viruses is associated with exposure to viral components such as virion surface glycoproteins and viral nucleic acids e.g. single (ss) and double stranded (ds) ribonucleic acid (RNA) (Alexopoulou et al. 2001; Diebold et al. 2004; Yoneyama et al. 2004). Viruses may induce changes in the host cells they infect, such as a reduction in surface multiple histocompatibility complex (MHC) expression. Virus infections are frequently associated with apoptotic cell death and lytic infections may also lead to release of intracellular contents such as heat-shock-proteins (hsp) into the extracellular environment (Basu et al. 2000). These signs of infection are detected by sentinel cells of the innate immune system, including the Langerhans cells or plasmacytoid or myeloid DCs in the tissues and bloodstream: ssRNA is believed to activate TLR7 (Diebold et al. 2004; Lund et al. 2004), dsRNA to activate TLR 3 (Alexopoulou et al. 2001) and DNA to activate TLR 9 if CpG motifs are present within the sequence (for DNA viruses) (Krug et al. 2004; Lund et al. 2003); viral glycoproteins are recognised by lectin receptors while hsp 70 activates both TLR 2 and TLR 4 (Asea et al. 2002). Sentinal cells both mediate effector activities and help to drive the activation of other components of the innate (and adaptive) immune response, for example, type 1 interferons produced by plasmacytoid DCs helps to activate cell types including DCs, NK cells and T and B cells.

Once activated, the innate response combats the infection directly via a variety of mechanisms including non-cytolytic pathways involving the actions of cytokines such as type 1 interferons (IFNs) and interferon-γ (IFN-γ) and via lytic pathways e.g. NK cell lysis of cells via perforin and granzyme-dependent mechanisms, as well as via the

activity of lytic cytokines such as tumour necrosis factor (TNF)-α (Kawai and Akira 2006; Le Bon and Tough 2002). The innate response also acts through cell-cell interactions e.g. the interaction of DCs with T cells and via secretion of cytokines e.g. type I IFNs, interleukins (IL)-12 and IL-15, to stimulate a predominantly type 1 adaptive response, including activation of cytotoxic CD8+ T cell and Th1 CD4+ helper T cell responses and the production of antibodies of a Th1-associated isotype appropriate to neutralise virions and eliminate virus-infected cells and clear the infection. $CD4^+$ T cells play a crucial role in the developing adaptive immune response by producing cytokines that direct immune function, sustain cytotoxic T lymphocyte (CTL) activity when required and potentiate CD8 T cell memory generation (Le Bon and Tough 2002; Pulendran and Ahmed 2006). CD8 T cells express T cell receptors (TCRs) that recognize specific 8 to 9-amino-acid-long peptides derived from viral proteins bound within a cleft in host MHC molecules expressed on the cell surface. Binding of the TCR to the peptide-MHC complex coupled with the appropriate maturation/activation signals activates the T cells, switching on a program of activation and proliferation that allows the activated CD8 T cell to clear the specific viral infection, usually through cell contact-dependent lysis of virus-infected cells and by production of antiviral cytokines such as IFN-γ (Roitt et al. 2003).

Different effector pathways or cell subsets play different roles in the control of different viral infection (Haller et al. 2006). For example, while type 1 IFNs are critical for early control of many viral infections, the activity of the NK cell subset is especially required to control herpesvirus infections (Yokoyama et al. 2004). $CD8^+$ T cells play a key role in eliminating many established viral infections. $CD4^+$ T cells mostly provide help to $CD8^+$ T cells but are important in clearing persistent

infections. Antibodies help to contain viral spread and are important for clearance of viral infections in the central nervous system (CNS) but their main role is frequently to mediate protection and reinfection.

1.3 Components of innate immune activation

There are many mechanisms for innate recognition of pathogen components, including Toll-like receptors, intracellular helicases such as RIG-I and NOD, C-type lectin receptors, mannose receptors, NK cell receptors and Fc receptors. Many of these receptors have evolved to become specialized (although not restricted) to recognizing particular subsets of pathogens and to stimulate the most appropriate response. As the study investigated the type 1 IFN response to LCMV, this review will focus on the the best characterized pathways for induction of type 1 IFN in response to viral pathogens, namely activation through the toll-like receptor and the intracellular helicase pathways.

1.3.1 Toll-like receptors in innate immunity

The family of TLRs are a series of transmembrane proteins that are highly conserved in many species, from the worm *C. elegans* to mammals. The prototype receptor, Toll, was first described in the fruit fly Drosophila, where it is involved in the development of embryonic dorsoventral polarity but also, more relevantly it was found to be critical for appropriate initiation of anti-fungal response in flies (Hashimoto et al. 1988; Lemaitre et al. 1996). TLRs play an important role in early host defence against pathogens. So far, 10 members of the TLR family have been identified in humans and 12 in mice (Akira and Takeda 2004; Akira et al. 2006; Medzhitov 2001). Each type of TLR recognizes a distinct subset of ligand(s). Synthetic ligands for all of the types of

receptors and some of the biological ligands for most of the TLRs have been identified.

All TLRs are type 1 integral membrane proteins which share a structural template consisting of extracellular domains containing varying numbers of leucine-rich-repeat (LRR) motifs and a cytoplasmic signalling domain that is homologous to the signalling domain of the interleukin 1 receptor (IL-1R), known as the TLR/IL-1R (TIR) domain. They thus activate same signalling adaptors used in IL-1-mediated signalling (Bowie and O'Neill 2000). The 24-29 amino acids in the LRR motif are arranged thus: XLXXLXLXX, sometimes with conserved residues, XØXXØXXXXFXXLX (F=phenylalanine, L=leucine, X=any amino acid, Ø=hydrophobic residue). The LRR domains are composed of 19-25 tandem LRR motifs; each LRR is folded into a β strand and an α helix connected by loops. As these receptors are germ-line encoded, there is a limit to the heterogeneity of ligands that can be recognized by TLRs. Recognition via TLRs is therefore focused on generic components of microbial pathogens which are difficult for the microorganism to alter. In line with this, biological ligands, or pathogan associated molecular patterns (PAMPs) so far identified for all the TLR members (listed in Table 1.1 and adapted from (Akira and Takeda 2004; Akira et al. 2006)) are components essential for the survival of the pathogen, ranging from structural molecules, e.g. peptidoglycan, LPS and mannan, to viral nucleic acids encoding genetic information. It should be noted that some TLRs also recognize host-derived components, such as single-stranded (ss) RNA or hsps.

Table 1.1 TLRs and their currently identified ligands (adapted from Akira et al, 2006)

Receptor	Ligand	Organism	Species
TLR1/TLR2	Triacyl lipopeptides	Bacteria	Bacteria and mycobacteria
TLR1/2	Soluble factors	Bacteria	*Neisseria*
TLR2	PG	Bacteria	Gram-positive bacteria
TLR2	Porins	Bacteria	*Neisseria*
TLR2	Lipoarabinomannan	Bacteria	Mycobacteria
TLR2	Phospholipomannan	Fungus	*Candida albicans*
TLR2	tGPI-mutin	Parasite	*Trypanosoma*
TLR2	Hemagglutinin protein	Virus	Measles virus
TLR2	ND	Virus	HCMV, HSV1
TLR2 and TLR4	Glucuronoxylomannan	Fungus	*Cryptococcus neoformans*
TLR3	dsRNA	Virus	Viruses
TLR4	Lipopolysaccharide (LPS)	Bacteria	Gram-negative bacteria
TLR4	Mannan	Fungus	*Candida albicans*
TLR4	Glycoinositolphospholipids	Parasite	*Trypanosoma*
TLR4	Envelope proteins	Virus	RSV, MMTV
TLR4	Heat-shock protein 60, 70	Host	
TLR4	Fibrinogen	Host	
TLR5	Flagellin	Bacteria	Flagellated bacteria
TLR6/TLR2	Diacyl lipopeptides	Bacteria	*Mycoplasma*
TLR6/TLR2	Lipoteichoic acid	Bacteria	Group B *Streptococcus*
TLR6/TLR2	Zymosan	Fungus	*Saccharomyces cerevisiae*
TLR7	Broprimine	-	Synthetic compound
TLR7	Loxoribine	-	Synthetic compound
TLR7/8	Imidazoquinolene (R848)	-	Synthetic compound
TLR7 and TLR8	ssRNA	Virus	RNA viruses
TLR9	CpG-DNA	Bacteria	Bacteria and mycobacteria
TLR9	Hemozoin	Parasite	*Plasmodium*
TLR9	DNA	Virus	Viruses
TLR11	Flagellin	Bacteria	Uropathogenic bacteria
TLR11	Profilin-like molecule	Parasite	*Toxoplasma gondii*

The structure of human TLR3 was recently elucidated and it suggests, contrary to previous predictions that the extracellular LRR domain would form a horseshoe structure with the ligand binding to the concave surface, that the ligand, which is negatively charged dsRNA, is more likely to attach to the convex surface of the domain (Choe et al. 2005). However, some TLRs are able to recognize PAMPs with different structures. For example, TLR4 is activated by structurally unrelated molecules like LPS, the plant diterpene paclitaxel, the fusion protein of respiratory syncytial virus, fibronectin and heat-shock proteins (Akira and Takeda 2004; Kawasaki et al. 2000; Kurt-Jones et al. 2000; Vabulas et al. 2002). This may be due to the presence of adaptor proteins such as MD-2 which interact with selected TLRs to provide the flexibility of recognition (Shimazu et al. 1999).

TLRs can be divided into several subfamilies, each of which recognizes related PAMPs – in general, lipids are recognized by TLR1, TLR2, and TLR3 whilst nucleic acids are recognized by TLR3, TLR7 and TLR9.

In the host, TLRs have a wide tissue distribution and are expressed on both immune cells such as dendritic cells, macrophages, mast cells, neutrophils, B cells, T cells and other cell types such as fibroblasts and epithelial cells. Expression is modulated rapidly in response to pathogens, certain cytokines and environmental stresses. As some of the biological ligands are expressed by the host, e.g. ss RNA, sequestration of TLR into distinct subcellular compartments where the host molecules would not be normally present is used to allow discrimination between host and pathogen. For example TLR7 is sequestered in the endocytic compartment, while host mRNA is typically localized in the nucleus. As viral replication and transcription in the host cell often occurs outside the nucleus but within the cytoplasm, ssRNA encountered by

TLR7 in the endocytic compartment would be likely be virally derived (Barton et al. 2006; Diebold et al. 2004).

Signalling via the TLRs on innate cells can trigger direct antiviral activity and promote the induction of further innate and also an adaptive immune response. For example, activation of TLR 3 or 9 on human NK cells by dsRNA or CpG in the presence of IL-12 leads to secretion of high amounts of IFN-γ and TNF-α while IFN-α secreted in response to TLR3, TLR7 or TLR9 activation upregulates interferon responsive genes with direct antiviral activity such as 2'5' oligoadenylate synthetase (OAS) (Banchereau et al. 2000) and also mediates the activation of innate subsets including DCs and NK cells and promotes the development of the adaptive immunity. Triggering of distinct TLRs on DCs elicits different cytokine-production profiles and different immune responses (Pulendran 2005; Pulendran and Ahmed 2006).

1.3.1.1 TLR signalling and its activation during viral infection

TLRs and the IL-1R share common signalling pathways. Stimulation with their ligands recruits TIR-domain-containing adaptors including myeloid differentiation marker 88 (MyD88) and TIRAP to the receptor, leading to the formation of a complex of IL-1R-associated kinases (IRAKs), tumour necrosis factor receptor-associated factor 6 (TRAF6) and interferon regulatory factor (IRF)5 (Takaoka et al. 2005; Taniguchi et al. 2001), as outlined in Figure 1.1. TRAF6 functions as an E3 ubiquitin ligase enabling it to catalyze the K63-linked polyubiquitin ligase complex of UBC13 and UEV1A. This activates the TGF-β activated kinase (TAK)1 complex, resulting in the phosphorylation of IκB kinase (IKK)γ/ nuclear-factor κB (NF-κB) essential modulator (NEMO) and activation of IKK complex (Mizukami et al. 2002; Takaesu et al. 2000). Phosphorylated IκB undergoes K48-linked ubiquitination and Fig1

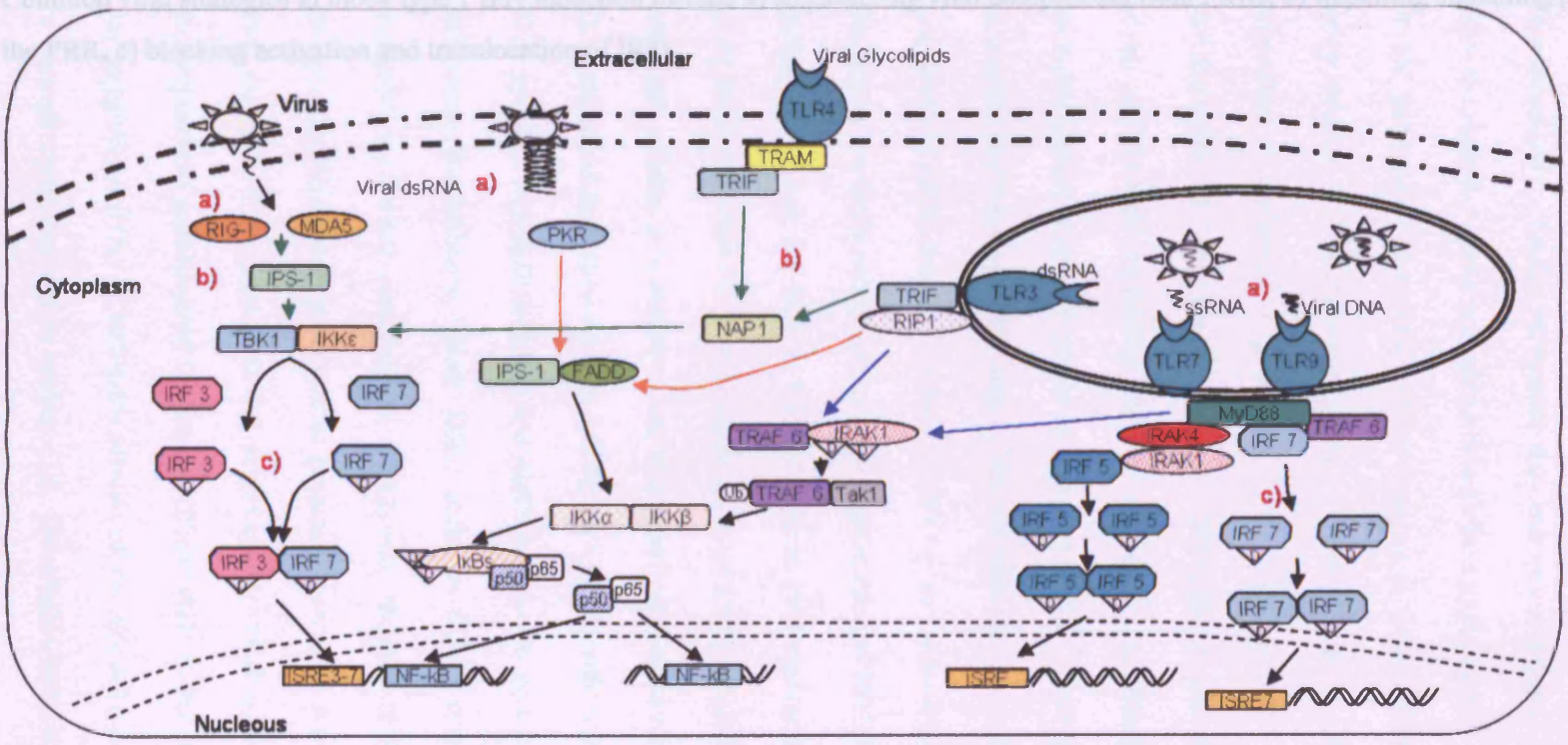

Figure 1.1 Pathways involved in the transcriptional activation of type 1 IFN gene expression following virus infection and viral counter-acting strategies

Overview of the signalling pathways leading to type 1 IFN production so far described are explained. Abbreviations are explained in the text. (adapted from Kawai and Akira, 2006)

Common viral strategies to block type 1 IFN induction include a) sequestering viral components from PRRs, b) impairing signalling pathway of the PRR, c) blocking activation and translocation of IRFs.

degradation by the proteosome, allowing NF-κB to translocate into the nucleus to initiate the expression of proinflammatory cytokine genes (Deng et al. 2000). Simultaneously, TAK1 activates the mitogen-activated protein (MAP) kinase cascades, leading to the activation of activator protein (AP)-1, which is also critical for the induction of cytokine genes (Wang et al. 2001). TLR4 triggers the MyD88-independent, TIR-domain-containing adaptor-inducing IFN (TRIF/TICAM-1)-dependent signalling pathway via TRIF-related adaptor molecule (TRAM/TIRP/TICAM2) to induce type 1 IFNs (Hoebe and Beutler 2004; Yamamoto et al. 2002).TRIF activates NF-κB and IRF3, resulting in the induction of proinflammatory cytokine genes and type 1 IFNs. TRAF6 and the death domain serine/threonine kinase RIP1 induce NF-κB activation and TRAF-associated NF-κB activator (TANK)-binding kinase (TBK1) or inducible IκB kinase (IKK-I) phosphorylate IRF3, which induces the translocation of IRF3.

Biological ligands for TLR 3, 7 and 9 include viral nucleic acids and these are thus likely to be the main TLRs responsible for type 1 IFN production in response to viral infections. There is a certain level of redundancy and overlap in the potential for TLR-mediated activation of type 1 IFN, as a virus could possess ligands for several TLR species, e.g both ssRNA and dsRNA may be generated during RNA virus infections. Furthermore, other TLR, such as TLR2, may also be involved in recognition of viral components. TLR2 was thought to be mainly involved in recognition of bacterial pathogen as peptidoglycan is its main biological ligand, but was recently also shown to be activated by viral glycoproteins such as the paramyxovirus glycoprotein (Oldstone 2006) while it has been suggested that the haemagglutinin (HA) of influenza viruses of the H2 subtype may activate innate B cell lymphocytes through an orphan TLR (Marshall-Clarke et al. 2006).

As briefly outlined above, TLR7, 8 and 9 signal through MyD88 to form a complex with IRAK4 and TRAF6 and also IRF7. IRF7 becomes phosphorylated by IRAK-1, translocates into the nucleus and activates expression of multiple IFN-α genes. This leads to autocrine induction of IRF7 and IRF8 which ultimately generates a positive feedback loop for high production of IFN-α subtypes and IFN-β (Asselin-Paturel and Trinchieri 2005; Tsujimura et al. 2003). This pathway is critical for induction of type 1 IFN in plasmacytoid DCs during the initial stages of a viral infection. Robust production of IFN-α/β via this pathway in plasmacytoid DCs is largely due to high pre-existing levels of IRF7 protein in the cytoplasm which helps kick-start the pathway and amplify the response to the initial danger signal. IRF7 serves as the master regulator of type 1 IFN production (Honda et al. 2005a). Other cells do not usually express IRF7 in a constitutive manner and the reason plasmacytoid DCs are such rapid producers of type 1 IFNs may stem from the presence of constitutively high levels of IRF7, coupled with expression of the appropriate TLRs, namely TLR7 and TLR9, that allow this DC subset to sense a viral pathogen and generate a swift and appropriate anti-viral innate response. Also, it was recently noted that within plasmacytoid DCs, ligand-bound TLR9 (and presumably also TLR7) remain active in the endosomal compartment for a longer period compared to other cell types, where after activation, ligand-bound TLR9 is rapidly translocated to lysosomes for degradation. This would again serve to amplify the type 1 IFN response in plasmacytoid DCs.

The MyD88-dependent pathway controls the expression of genes required for inflammatory and adaptive immune responses, such as IL-6, IL-1β, tumour necrosis factor (TNF) and IL-12p40 (Kaisho et al. 2001; Kawai et al. 1999; Kawai et al. 2001).

IRAK-1 also induces phosphorylation of IRF7, which dimerizes and translocates into the nucleus where it induces type 1 IFN gene expression.

1.3.2 Recognition of intracellular infection by RNA helicases

Most cells in the body contain intracellular sensors that upon infection with an RNA virus, detect viral components, usually single or double stranded RNA and activate IRF3 and NF-κB which in turn activate IFN-β expression. Infected cells initially secrete IFN-β and IFN-α4 but then the positive amplification feedback loop upregulates expression of other IFN-α subtypes.

Fibroblast cells lack expression of TLR3, 7 or 9. Instead, these and most cells in the body express two cytoplasmic helicases, RIG-I and MDA5, that detect intracellular infection via the presence of dsRNA, a common replicative intermediate during viral replication and transcription. RIG-I and MDA5 apparently bear non-redundant roles but may have specificity for certain viruses (Kato et al. 2006; Yoneyama et al. 2005). RIG-I and MDA5 both recognize polyIC, a synthetic dsRNA analogue; however, RIG-I recognizes *in vitro*-transcribed dsRNA while MDA5 does not (Kato et al. 2006). Likewise, studies using RIG-I and MDA5 knock-out mice have shown that RIG-I is essential for production of type 1 IFN in response to influenza virus, whereas MDA5 is critical for picornavirus detection.

RIG-I and MDA5 are related, containing two N-terminal caspase recruiting domain (CARD)-like regions and a C-terminal DExD/H box RNA helicase domain (Andrejeva et al. 2004; Yoneyama et al. 2005). It is proposed that interaction between the helicase and its ligand dsRNA induces a conformational change which allows the CARD domain to interact with CARD-like domains of downstream signalling partners, one of which was independently isolated by a number of research groups and

is known variously as interferon-β-promoter-stimulator 1 (IPS-1), mitochondrial antiviral signalling molecule (MAVS), virus induced signalling adaptor (VISA) or CARD adaptor inducing IFN-β (CARDIF) (Kawai et al. 2005; Meylan et al. 2005; Seth et al. 2005; Xu et al. 2005) (in the rest of the thesis the protein will be referred to as IPS-1).

Marques *et al* have put forward a theory that these helicases discriminate between self (microRNAs) and viral dsRNA according to the type of end structure of the RNA rather than the sequence; 2 nucleotide (nt) overhangs on the 3' end of short lengths (~21-27 nt) of duplexed RNA activated the helicases, while blunt ended duplexed RNA of similar length, which are structurally analogous to self-microRNAs, did not substantially activate helicase activity (Marques et al. 2006).

As shown in Figure 1.1, the adaptor IPS-1 bridges the triggering of the helicases with NF-κB activation – IPS-1 too posses a CARD-like domain that binds activated RIG-I or MDA5 but it is able to interact through its C-terminal domain with FADD and RIP1 which are able to activate NF-κB and turn on transcription of pro-inflammatory cytokines, including IFN-β and other interferon stimulated genes. IPS-1 is a mitochondrial associated protein, which localizes to the outer mitochondrial membrane through its C-terminal transmembrane domain. It has been suggested that this may keep the interacting RNA-sensing helicases close to sites of viral replication inside the cell.

Direct activation of type 1 IFN is also mediated by IPS-1; it activates IRF3 by a yet unidentified mechanism, which is believed to involve recruitment of two Iκβ kinase (IKK)-related kinases, Iκκε and TBK-1, known to phosphorylate IRF3 and IRF7. Phosphorylated IRF3 and IRF7 then translocate into the nucleus to upregulate IFNα/β expression (Kawai et al. 2005; Seth et al. 2005). This would suggest that the TLR and

RIG-I signalling pathways converge at the level of Ikkε and TBK-1 and that both pathways evolved for a similar purpose – rapid upregulation of type 1 IFN.

A similar CARD-dependent signalling pathway exists for the detection of foreign lipid molecules. Nucleotide oligomerization domain (NOD) proteins represent another set of cytplasmic pathogen sentinels. NOD1 and NOD2 are believed to recognize bacterial components. Like RIG-1 and MDA5, the NOD proteins contain N-terminal CARD domains and signal upregulation of NF-κB and corresponding IFN α/β amplification.

Another helicase, LGP2, has been identified that shares sequence similarity with RIG-I in the helicase domain but lacks the CARD signalling intercellular region. It has therefore been proposed that LGP2 may act as a negative regulator of the RIG-I and MDA5 signalling (Rothenfusser et al. 2005; Yoneyama et al. 2005).

It has been suggested that a viral DNA-specific helicase also exists, which induces production of type 1 IFN via the IPS-1 and TBK1/ Ikkε pathway (Stetson and Medzhitov 2006). The protein has yet to be identified, but its existence is highly likely as previous experiments showed that herpes simplex virus (HSV) induces type 1 IFN in a both TLR9-dependent and TLR9-independent manner (Malmgaard et al. 2004).

1.4 The Type 1 IFN Response

1.4.1 Overview

Interferons (IFNs) were first identified in the 1950s on the basis of their ability to protect cells from virus infection (Isaacs and Lindenmann 1957). IFNs belong to a large family of cytokines that exert pleiotropic effects, especially in the generation of antiviral immunity. They are part of the class II family of cytokines that also encompasses IL-10 and IL-10 homologs (IL-19, IL-20, IL-22, IL-24, IL-26) (Donnelly et al. 2004; Kotenko et al. 2003; Pestka et al. 2004). IFNs are further grouped into two major classes based on their biological activity, antigenicity and the cell types from which they are secreted (Le Page et al. 2000a): type 1 IFNs (IFN-α, -β, -δ, -ε, -κ, - ϖ ,-τ and –ζ (also called limitin)) which are stable at pH 2.0 and type 2 IFN (IFN-γ) which is inactivated at pH 2 (Boxaca M 1967).

Type 1 IFNs are derived from a single ancestral gene. The multiple IFN-α genes are believed to have arisen through repeated gene duplication and recombination; subtle modifications of their promoter and regulatory sequences allow them to be differentially expressed depending upon the cell type, stimulating agent and environment (De Maeyer and De Maeyer-Guignard 1998). IFN-α and -β are the best studied members within the type 1 IFN family. In mice, there at least 10 distinct *IFN-α* genes clustered within the *Ifna* locus on chromosome 4 that encode biologically active IFN-α subtypes. These are intronless genes with a high degree of homology (80-95%) that produce proteins of 16-27kDa which are 166 residues in length and are sometimes glycosylated (Doly et al. 1998; Le Page et al. 2000a; van Pesch et al. 2004). In contrast, only one gene (*IFN-β*), also intronless and located on chromosome 4, encodes the IFN-β protein. IFN-β is a glycosylated protein of 28-35kDa containing

166 amino acid residues. All type 1 IFNs signal through a common receptor, the type 1 IFN receptor (IFN-R).

Some type 1 IFNs are species or cell specific e.g. IFN-δ is found only in porcine species, IFN-τ is expressed in ruminants such as cattle, sheep and giraffes (Liu et al. 1996), limitin is only expressed in mice while IFN-κ is only expressed by keratinocytes.

A subset of type 1 IFNs appear not to function primarily as anti-viral cytokines. IFN-δ and IFN-τ, termed trophoblast IFN, are important during pregnancy, where they are expressed at high levels by the trophoblast as an antileukocyte protein to allow proper implantation of the ovum. Additionally, trophoblast IFN has been reported to posses immunosuppressive effects against $CD4^+$-lymphocytes. IFN-ϖ and IFN-ε are expressed by humans and are also reported to play a role in maternal recognition of pregnancy, suggesting they may be the human equivalent of IFN-τ. Promoter analysis of the bovine IFN-τ gene indicates that while expression may still be induced by viruses, it also contains elements for trophoblast-specific expression (Cross and Roberts 1991). Thus, unlike IFN-α and -β, these IFNs have limited anti-viral activity and likely have a physiological role as pregnancy hormones. As this study focuses on the antiviral effects of interferon, the term type 1 IFNs will be used in the rest of the text to denote IFN-α and IFN-β, whose antiviral activities have been most investigated.

Type II interferon or IFN-γ is encoded by a gene on chromosome 10 in mice and 12 in humans. The IFN-γ gene contains 3 introns; it encodes a protein of 143 amino acids in length. The translated protein, which has a typical molecular mass of 20-25kDa, shares little structural homology with the type 1 IFNs. IFN-γ is mainly secreted by

NK cells and activated, mature cytotoxic T cells again in response to viral infections. IFN-γ binds to a single receptor, consisting of two chains, IFNγR1 and IFNγR2.

A new class of interferon-like molecules have been recently described in a number of species including humans and mice; these cytokines, which are distantly-related to the classical type 1 IFNs (15-19% amino acid identity with IFN-α) are grouped under a new interferon type, IFN-λ, of which 3 subtypes have so far been identified - IFN-λ1, IFN-λ2, IFN-λ3 (also known as IL-29, IL-28A and IL-28B). Unlike other members of the IFN family, which in humans are intronless genes located on a locus on chromosome 9, the genes encoding IFN-λ subtypes contain introns and are clustered on chromosome 19 (Sheppard et al. 2003). These cytokines, which may be provisionally termed type III IFNs, signal via a separate cell-surface receptor composed of two chains, IL-28Rα and IL-10Rβ (Donnelly et al. 2004). While the IFN-λ proteins are structurally related to the type 1 IFN proteins, their genomic organization shares many similarities with the IL-10 family, leading some researchers to hypothesize that these cytokines may represent an evolutionary link between type IFNs and the IL-10 family (Sheppard et al. 2003).

Within the context of immunity, type 1 IFNs are known to induce potent anti-viral effects as well as anti-tumour activity and apoptosis. Furthermore, they are important mediators in the modulation of the adaptive response. Type 1 IFNs mediate their activity via the upregulation of effector molecules that directly influence protein synthesis, cell growth and survival. Microarray analysis has shown that signalling of type 1 IFNs through the IFN-R modulates expression of hundreds of genes (Baechler et al. 2003; Der et al. 1998).

Studies in mice treated with neutralizing antibodies against IFN-α and IFN-β or in mice with an IFN-R knock-out (k/o) phenotype showed impaired upregulation of type

1 IFN when exposed to viruses, indicating that constitutive expression of type 1 IFN appears to be a pre-requisite for enhancement of type 1 IFN production after viral infection (Hata et al. 2001). Pre-existing levels of type 1 IFN are also needed for efficient induction of IFN-γ signalling.

Type I IFN can be produced by most cells if they are virally infected, and is also produced by specialised cells of the innate immune system in response to stimuli associated with viral infection (Doly et al. 1998), (Biron 2001). It used to be thought that IFN-α is mainly secreted by peripheral blood leukocytes (plasmocytoid DCs, other DC subsets, macrophages, monocytes and lymphoblastoid cells e.g. B lymphocytes) while epithelial and fibroblastic cells are the main secreters of IFN-β. However, it now appears that all cell types secrete all subtypes of IFN-α or IFN-β, if only in varying proportions. Viral infections are believed to be the most potent natural inducers of type 1 IFN. IFN-mediated protection occurs within hours of viral infection (Le Page et al. 2000a).

1.4.2 Pathways leading to production of IFN-α/β protein

Type 1 IFNs are among the earliest cytokines secreted by the host following viral infection. They can be produced so quickly, as reviewed above, because the transcription of type I IFN genes can be activated by a host of pathogen sensing receptors located inside and outside the cells, via a number of intracellular phosphorylation pathways that are parallel, convergent or completely independent of each other, but that ultimately result in translocation of the transcription factors NF-κB, IRF3 or IRF7 into the nucleus where they bind IFN-α or -β genes to initiate transcription (Beutler et al. 2006; Doly et al. 1998; Honda et al. 2005b; Theofilopoulos et al. 2005).

Type 1 IFN genes contain multiple independent promoter elements, termed positive regulatory domains (PRD or viral recognition elements (VRE) which are recognized by multiple transcription factors (Civas et al. 2002; Doly et al. 1998; Goodbourn and Maniatis 1988; Leblanc et al. 1990; Mamane et al. 1999; Sato et al. 2001b; Taniguchi et al. 2001). The protein coding sequences of all the type 1 IFN genes are not interrupted by introns (Kelley et al. 1985; Kelley and Pitha 1985a; b; Shaw et al. 1983; Vodjdani et al. 1985) thus doing away with the need for post-transcriptional processing, enabling speedy transcription and translation of the proteins.

IFN-β and IFNα-4 are produced initially, then they upregulate both their own production and that of other IFN-α subtypes by a positive feedback mechanism (Le Page et al. 2000a) through activation of the transcription factors IRF3 and IRF7.

The 'first wave' of type 1 IFN is often insufficient to control a viral infection. However, in order to generate the 'second wave', categorised by transcription of additional subtypes of IFN-α, the cell requires IRF7, which is one of many antiviral proteins synthesized in response to the first wave of IFN-α4 and IFN-β, thus generating a positive feedback loop. The same kinase complex, TBK1 IκB kinase ε (Sharma et al. 2003) is responsible for phosphorylation of both IRF3 and IRF7 and they themselves, along with pathogen sensing receptors such as TLRs and RNA helicases, are also IFN-inducible proteins, as are the ISGF3 components STAT1 and IRF9 needed to mediate production of IFN-stimulated antiviral proteins. All of these are therefore produced in response to the first wave of IFN production, and constitute both a positive feedback loop that not only induces and amplifies type 1 IFN secreted through the second wave, but also amplifies induction of anti-viral proteins (Mamane et al. 1999; Platanias 2005; Theofilopoulos et al. 2005). Failures in the activity of any of the key intermediary proteins will result in either complete abolition of type 1 IFN

production or a partial response with no ability to generate the second wave of IFN production. For example, when challenged with a respiratory viral infection, mice that lack STAT1 are only limited to production of the immediate-early IFN subtypes IFN-α4 and IFN-β and cannot mount positive feedback or express other IFN-α subtypes (Marie et al. 1998). Here, impaired IFN production in concert with impaired IFN responsiveness contributes to the exquisite sensitivity of STAT1-deficient mice to viral infection.

1.4.2.1 IRFs in type 1 IFN production

IRFs are transcription factors possessing a helix-turn-helix-DNA binding motif (Sato et al. 2001b; Taniguchi et al. 2001). At least nine type 1 IRFs have been identified (IRF1 to 9), which under normal conditions are resident in the cytoplasm. All IRFs characterised so far contain a conserved N-terminal binding domain which recognizes a consensus DNA sequence known as the interferon stimulated response element (ISRE) (Darnell et al. 1994; Tanaka et al. 1993). They are activated by phosphorylation, and gaining of the extra phosphate moiety allows the IRFs to translocate into the nucleus and initiate transcription of IFN-β and IFN-α subtypes. Lymphocytes express IRF3, IRF4 and IRF5 constitutively. Additionally, in plasmacytoid DCs, IRF7 is also constitutively expressed (Honda et al. 2005a; Prakash et al. 2005). Other IRFs are ubiquitously present and are themselves induced by type 1 IFNs.

Studies in mice lacking expression of selected IRFs demonstrated the importance of these signalling molecules to the development, regulation, and function of the innate and adaptive immune system. $IRF1^{-/-}$ mice had defective NK cells, NKT cells and intraepithelial $\gamma\delta^{+}$ T cells, believed to stem from the lack of IL-15 (Ogasawara et al.

1998; Ohteki et al. 1998). Additionally, these mice were unable to mount Th1 responses as T cell signalling was also dysregulated, affecting $CD8^+$ thymocyte development and positive and negative selection (Lohoff et al. 1997; Matsuyama et al. 1993; Penninger et al. 1997; Taki et al. 1997). When IRF2 expression was deleted, NK cell development and Th1 differentiation were again impaired, indicating that IRF1 and IRF2 both may be needed in a non-redundant fashion in order to allow normal NK and T cell development (Lohoff et al. 2000; Lohoff et al. 2002). IRF-$4^{-/-}$ mice suffered splenomegaly, lymphadenopathy, reduced humoral responses and serum Ig levels, and defective Th1 and Th2 differentiation (Lohoff et al. 2002; Mittrucker et al. 1997). Mice that lacked IRF8 were unable to respond to signalling via TLR3 and TLR9 (Schiavoni et al. 2002; Tsujimura et al. 2004; Tsujimura et al. 2003). Furthermore, plasmacytoid DCs were absent in lymphoid organs and there was a reduction in the number and activation state of $CD8\alpha^+$ DCs.

At least 4 members of the IRF family were initially believed to play a role in regulating type 1 IFN production. These were IRF1, IRF3, IRF5 and IRF7 (Barnes et al. 2001; Darnell et al. 1994; Mamane et al. 1999; Miyamoto et al. 1988; Nguyen et al. 1997; Taniguchi et al. 2001). However, further studies indicated that IRF1 and IRF5 were not essential for IFN-α or IFN-β gene induction. Type 1 IFN production in IRF$1^{-/-}$ embryonic fibroblasts (MEF) after infection with Newcastle disease virus (NDV) was not markedly different to that observed in normal MEF (Matsuyama et al. 1993). Similarly, MEFs from IRF$5^{-/-}$ mice infected with virus or stimulated with dsRNA produced normal levels of type 1 IFN (Takaoka et al. 2005). However, IRF5 is thought to be of importance in regulating pro-inflammatory cytokines via an MyD88-TRAF6-NF-κB dependent manner. Although some of the pathway remains to be uncovered, what is thus far known is that cytoplasmic IRF5 interacts with MyD88

and TRAF6 and becomes activated through a mechanism that is not yet clear. Activated IRF5 then translocates into the nucleus where it interacts or co-operates with activated NF-κB to switch on transcription of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12. This conclusion was made following observations in mice which were deficient in IRF7; while they were unable to produce IFN-α or IFN-β in response to TLR-activation, they were still able to produce normal levels of IL-12 and IL-6, suggesting these cytokines are induced in an MyD88 and NFκB –dependent but IRF7-independent manner (Honda et al. 2005a).

1.4.2.2 IRF3

IRF3 is expressed constitutively in a variety of cells where it is present in the cytoplasm as a monomer (Lin et al. 1998b; Lin et al. 1999; Sato et al. 1998; Suhara et al. 2000; Wathelet et al. 1998; Yoneyama et al. 1998). IRF3 has potential phosphorylation sites in the C-terminal region (Ser385, 386, 398, 402 and 405, and Thr404 of human IRF3) however, to date, only one site has been conclusively shown to be phosphorylated (Ser386) (Mori et al. 2004; Servant et al. 2003). Phosphorylation induces homodimerization and activation, which enables the dimerized proteins to translocate into the nucleus where they form a complex with a co-activator p300/CBP (Hiscott et al. 1999; Suhara et al. 2002). The complex then binds the PRDI or PRDIII sequence on the promoter region of the IFN-α or IFN-β genes (Lin et al. 1999; Servant et al. 2003; Suhara et al. 2000). Other non-IFN genes such as RANTES and IP10 have also been reported to be induced by IRF3. IRF3 phophorylation is mediated by TBK1 and IKKε kinases (Sharma et al. 2003) and as such occurs as a consequence of activation of TLR4, TLR3 or RIG-I/MDA5 (see Figure 1.1). IRF3 is

therefore an important adaptor in the initial production of type 1 IFN during the early stages of viral infection.

1.4.2.3 IRF7

Like the other IRFs described, IRF7 is found as a monomer in the cytoplasm. Activation requires phosphorylation of the C-terminal serine residues, followed by dimerization (Lin et al. 2000; Marie et al. 1998; Marie et al. 2000; Sato et al. 1998). Sharma et al demonstrated that IRF3 and IRF7 were phosphorylated by the kinases IKKε and TBK1, which are themselves activated by viruses or dsRNA acting through TLR3/TLR4 or RIG-I/MDA5 (Fitzgerald et al. 2003; Sharma et al. 2003). In murine IRF7, Ser437 and 438 are the primary targets for phosphorylation; although other residues have been identified, namely Ser425-426, Ser429-431 or Ser441IRF7, as possible partners (Caillaud et al. 2005). Additional phosphorylation is needed to fully activate IRF7 in virus-infected cells, suggesting that it serves as a main transfer molecule for type 1 IFN-mediated signalling from uninfected or infected cells. Activated IRF7 subsequently induces production of IFN-α subtypes and IFN-β (Nakaya et al. 2001; Sato et al. 1998; Sato et al. 2000) (Marie et al. 1998).

Honda *et al* provided evidence of IRF7 being the essential regulator of type 1 IFN production in all cell types, including plasmacytoid DCs and conventional DCs, with IRF3 being dispensable for IFN-α production by viral activation of TLR7, TLR8 or TLR9 (Honda et al. 2005a). Studies they carried out with knock-out mice showed that in MEFs generated from IRF7 deficient mice, IFN-α/β expression was more severely impaired after infection with vesicular stomatitis virus (VSV), HSV and encephelomyocarditis virus (EMCV) than responses in $IRF3^{-/-}$ MEFs. Viral infection of IRF7 and IRF3 knock-out mice further suggested that IRF7-deficient mice do not

produce much serum IFN and are more susceptible to viral infection than IRF3-deficient mice. The IRF7-dependent pathway may therefore be a critical regulator of type 1 IFN production, able to promote IFN production in $IRF3^{-/-}$ cells. In contrast, in $IRF7^{-/-}$ mice with normal IRF3 expression, the absence of IRF7 abolishes the type 1 IFN response. The authors of the study hypothesized that IRF7 may be present in low levels in uninfected cells, probably synthesized in response to low levels of IFN-α pre-existing in the cell. This low-level IRF7 or IFN-α/β production occurred in the absence of functional IRF3. By contrast, while IRF3 contributes to type 1 IFN induction, it may need to interact with IRF7 to function. In other words the homodimers of IRF7 or heterodimers of IRF7 and IRF3, rather than IRF3 homodimers may be responsible for inducing IFN-α/β in MEFs infected by viruses. Once the initial activation of IFN genes is achieved by IRF7 (and IRF3), the positive-feedback regulation becomes fully operational, wherein IFN-induced IRF7 participates (Honda et al. 2005b).

The importance of feedback and the role of IRF7 were investigated by Kalinke's group (Barchet et al. 2002), who conducted studies measuring IFN-α production by splenocytes lacking the IFNR in response to VSV or ultraviolet (UV)-inactivated HSV. They could not detect any difference in the levels of IFN-α induced nor upregulation of IRF7 in the spleen of $IFNR^{+/+}$ and $IFNR^{-/-}$ mice, and so concluded that early IFN production *in vivo* did not require IFN feedback amplification or IRF7 (Barchet et al. 2002). However, others have suggested that the route of infection may influence the responsive cell type and hence the results obtained (Levy 2002). Systemic infections (e.g. intra venous (i.v)) may lead to efficient activation of splenic plasmacytoid DCs while peripheral infections may only target localised responsive cell types. In accordance with this hypothesis, Prakash *et al* reported that when using

an RNA virus infection model inducing a localised as opposed to systemic infection by targeting the lungs, they found that IFN production correlated with IRF7 production in the lungs of normal mice, and STAT1 deficient mice that were unable to upregulate IRF7 exhibited very impaired type 1 IFN production (Prakash and Levy 2006). Analysis of the tissue distribution of IRF7 showed that most mouse tissues expressed IRF7 in an induction dependent manner, i.e. not constitutively, except for plasmacytoid DCs which had very high pre-existing levels of IRF7 protein and consequently were able to respond rapidly to virus infection by robust production of multiple IFN-α species (Marie et al. 2000). These observations emphasise that differential regulation of IRF7 expression constitutes the underlying mechanism of positive-feedback regulation in peripheral tissues and is an important element of cell-type specific differences in IFN production.

In addition to secreting normal levels of pro-inflammatory cytokines IL-12 and IL-6, IRF7-deficient mice exhibit normal activation of other adaptors such as NF-κB, JNK and p38 (Honda et al. 2005a). This implies that IRF7 has a specialised role in regulating only type 1 IFN transcription within the MyD88-dependent cytokine gene induction program activated by TLR signalling. The function of the cytoplasmic molecular complex anchored by MyD88 in the activation of NF-κB/MAP kinases is therefore under separate control and signalling, so that arm may be modified without affecting the regulation of type 1 IFN induction.

Studies with IFN-$\beta^{-/-}$ mice have confirmed the role played by IFNβ in the induction of the overall IFN response. In IFN-$\beta^{-/-}$ mice, the expression of various IFN-α transcripts and IFN inducible genes (2'-5'OAS) were much lower compared to heterozygous or wild type control after viral infection, rendering the k/o mice more susceptible to vaccinia infection (Deonarain et al. 2000). Subtypes of IFN-α are also important in

viral control. IFN-α2, -α5, -α6 and -α8 subtypes were found to be induced specifically in response to NDV infection by IRF7-mediated activation (Levy et al. 2003b; Marie et al. 1998).

1.4.3 Type 1 IFN Signalling

1.4.3.1 The type 1 IFN receptor

Type 1 IFNs have direct antiviral effects and also regulate the activation, proliferation and differentiation of multiple cell types, thus affecting both innate and adaptive immune responses. IFNs are signalling molecules and achieve their diverse effects through activation of a single cell-surface receptor, the IFN-R, triggering intracellular signalling pathways that result in upregulation of the transcription of IFN-inducible genes. The IFN receptor is broadly expressed at low levels, (between 200-6000 copies per cell) and binds IFNs secreted into the extracellular environment with high affinity (dissociation constant of 10^{-9}-10^{-10}) (Theofilopoulos et al. 2005).

While multiple subtypes of type 1 IFN exist, they bind and activate a single receptor, the IFN-R. One explanation for this incongruity may be because all the genes in the type 1 IFN subset are thought to be derived from a single ancestral gene, amplified through gene duplication and recombination events over time. The receptor is composed of two separate chains, IFNAR-1 and IFNAR-2. In humans and mice, IFNAR-1 consists of 590 amino acids, and has a mass of ~66kDa. The extracellular N-terminal of the molecule contains two 200 amino acid domains, as seen in other members of the class II cytokine family. The locus encoding IFNAR-1 contains 11 exons and is situated on chromosome 21 in the human genome (Benoit et al. 1993; Lutfalla et al. 1992; Lutfalla et al. 1990; Uze et al. 1990). IFNAR-2 is 331 amino acids in length, with a mass of ~51kDa. NMR studies of its extracellular portion

indicate it folds into two fibronectin type III domains orientated perpendicularly to one another and linked by an α-helical 'hinge' (Chill et al. 2003).

IFNAR-1 contains two IFN binding sites which is postulated to contribute to the diversity of biological responses elicited by different IFN-α subtypes (da Silva et al. 2002). There is biochemical evidence which suggest that the IFN-α and IFN-β subtypes engage the receptor chains differently, e.g. IFN-α2 and IFN-β utilise different regions of the IFNAR-2 for signalling, triggering different signalling responses, leading to distinct effects on cell function (Chill et al. 2002; Chill et al. 2003; Domanski et al. 1998). NMR studies have suggested that the N-terminal binding domain of IFNAR-2 is flexible, allowing it to adapt to the different structures of its various ligands and that binding (to IFN-α2) causes 'tightening' of the entire chain, increasing its rigidity. This increase in receptor rigidity may initiate the intracellular IFN signalling cascade (Chill et al. 2002). Interestingly, a single aromatic tryptophan (W) side-chain in the binding site on IFNAR-2 (^{R}W100) may be responsible for specific recognition of IFN-β. Mutation of the ^{R}W100A position abolishes IFN-β activity, while IFN-α activity decreases only 4-fold (Piehler et al. 2000).

Attachment of type 1 IFNs to the IFN-R activates the Janus kinase signal transducer and activator of transcription (JAK/STAT) pathway, activating IFN stimulated genes, as illustrated in Figure 1.2. (Grandvaux et al. 2002; Le Page et al. 2000a; Nguyen et al. 2002b).

1.4.3.2 Classical JAK-STAT signalling pathway

Each chain of the IFN-R interacts with a member of the Janus activated kinase (JAK) family in the cytoplasm (Darnell et al. 1994; Ihle 1995; Platanias 2005) Figure 1.2

outlines a schematic representation of the JAK-STAT signalling pathway. IFNAR1 interacts with tyrosine kinase 2 (Tyk2) while IFNAR2 binds JAK1. The initial signalling step involves attachment of a monomeric type 1 IFN molecule to the binding sites of the IFNAR-1 and IFNAR-2 chains of the receptor on the extracellular end of the chains, bringing both chains in close proximity to form a heteromeric complex. This induces structural rearrangement of the cytoplasmic ends of the chain resulting in ligand–induced receptor activation (Chen et al. 2004; Darnell et al. 1994; Ihle 1995; Platanias 2005; 2003). This promotes autophosphorylation and activation of the associated JAKs, which in turn allows recruitment and activation of the transcription factor signal transducer and activator of transcription 2 (STAT2) to the IFNAR-2 chain, followed by STAT1 (Stark et al. 1998). Both STAT2 and STAT1 are activated through tyrosine kinase-mediated phosphorylation. Phosphorylated STAT1 and STAT2 are released, then associate with IRF9, also known as p48, (which is not phosphorylated) to form a complex called the IFN-stimulated gene (ISG) factor 3 (ISGF 3). The complex translocates into the nucleus and binds genomic DNA at promoter regions containing IFN-stimulated response element (ISRE) sequence motifs to initiate gene transcription. The genes transcribed include those encoding IFN-β and IFN-α as well as IRF7 and IRF3 (Levy et al. 2003a), proteins which promote autocrine induction and amplification of type 1 IFN expression. Additionally, activated STAT1 is also able to form homodimers of STAT1:STAT1 which is also able to translocate into the nucleus. These homodimeric complexes bind to genomic DNA at promoter regions containing the γ-activated site (GAS) sequence motif to initiate gene transcription. Individuals with non-functional STAT1 succumb to viral infections, which likely reflects the importance of STATs in controlling viral infection through IFN signalling pathways (Dupuis et al. 2003).

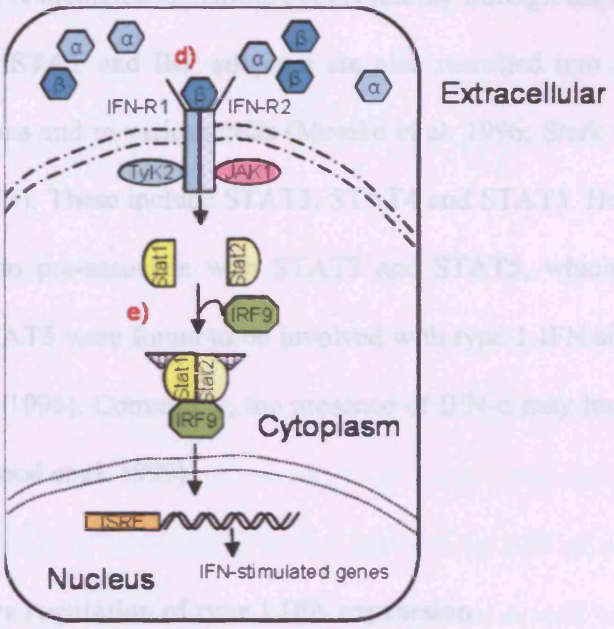

Figure 1.2 Activation of IFN-stimulated genes via the JAK/STAT pathway and viral counteracting strategies

Type 1 IFNs exert their biological functions by binding to the IFN-R, a cell surface receptor consisting of two chains, IFN-R1 and IFN-R2. This activates a series of transcription factors and kinases that make up the JAK/STAT pathway, which leads to induction of IFN-stimulated genes (ISG). The products of the pathway set up an antiviral, antiproliferative and immunoregulatory state in the cells (adapted from Honda et al, 2005). Unsurprisingly, this pathway is often targeted by viruses as a means to downregulate the antiviral effects of type 1 IFN production. Common viral strategies include d) preventing attachment of secreted type 1 IFN molecules to the IFN-R receptor and e) interfere with the activity of transcription factors of the JAK/STAT pathway.

While type 1 IFN-mediated signalling occurs mainly through the STAT2 and STAT1 adaptors, other STAT and IRF adaptors are also recruited into this pathway under certain conditions and in various cells (Meinke et al. 1996; Stark et al. 1998; van der Zeijst et al. 1983). These include STAT3, STAT4 and STAT5. However, STAT1 has been reported to pre-associate with STAT3 and STAT5, which may explain why STAT3 and STAT5 were found to be involved with type 1 IFN signalling complexes (Stancato et al. 1996). Conversely, the presence of IFN-α may inactivate STAT1 and STAT3 (Kirkwood et al. 1999).

1.4.4 Negative regulation of type 1 IFN expression

Excessive levels of type 1 IFN are detrimental to the host, and have been implicated in autoimmune disorders, toxicity, excessive inflammation and immunopathology. Even in non-toxic doses, prolonged systemic exposure to type 1 IFN may not be desirable. In patients with hepatitis C virus (HCV), long-term treatment with recombinant IFN-α is not without side effects, which may include autoimmune disorders such as insulin-dependent type 1 diabetes or rheumatoid arthritis; depression was another side effect. Patients with the condition systemic lupus erythematosus (SLE or lupus) often have increased levels of serum IFN-α that coincide with disease exacerbations (Bengtsson et al. 2000).

Mechanisms have therefore evolved within the host to downregulate type 1 IFN expression. As such, expression of type 1 IFN is typically followed by appropriate activation of ISGs as well as repression of further IFN activity and expression. Some of these mechanisms that negatively regulate type 1 IFN signalling include receptor internalization and degradation, induction of phosphatases to promote dephosphorylation of JAKs and STATs, expression of suppressors of cytokine

signalling (SOCS) (Fenner et al. 2006; van der Zeijst et al. 1983) and repression of STAT-mediated gene activation by protein inhibitors of activated STATs (PIAS) called small ubiquitin-like modifiers (SUMO) E3 ligases (Liu et al. 2004; Shuai 2003).

Within the human IFN-β gene, cis-acting elements of the IFN-β promoter such as negative regulatory domain (NRD)I and NRDII have been characterized as being involved in the constitutive repression of gene expression, while positive regulatory domains (PRDs) mediate both induction and repression (Whiteside et al. 1994) (Liu et al. 2004). Interplay of the binding of the activator as well as repression factors is necessary in regulating expression of type 1 IFN genes as well as for the differential expression of the IFN-α gene family.

The first IFN gene transcriptional repressor to be discovered was IRF-2, isolated by its homology to IRF-1 (Harada et al. 1989). IRF-2 binds PRDI and PRDIII within the viral regulatory element (VRE)-B of the IFN-β gene and also recognises the IRF elements present in other virus-inducible genes such as IFN-A (Au et al. 1992; Au et al. 1993) (Figure 1.3 A). This was confirmed when mice deficient of IRF-2 expression produced higher levels of IFN-A and IFN-β mRNA upon viral induction (Matsuyama et al. 1993). Furthermore, IRF-2 competes with the transcription factor IRF-1 for the IRF-binding site in the promoter region. Expression of IRF-2 is constitutive in many cell types and is believed to play a role in maintaining the gene repressive state before viral induction occurs. Type 1 IFNs up regulated in response to viral infection also induced IRF-2 expression and thus also repressed type 1 IFN gene expression post-induction (Harada et al. 1989; Lin et al. 1999; Whiteside et al. 1994).

Another repressor of genes containing PRD promoters is the IFN consensus sequence-binding protein (ICSP), which is a member of the IRF family (IRF9). Expression of

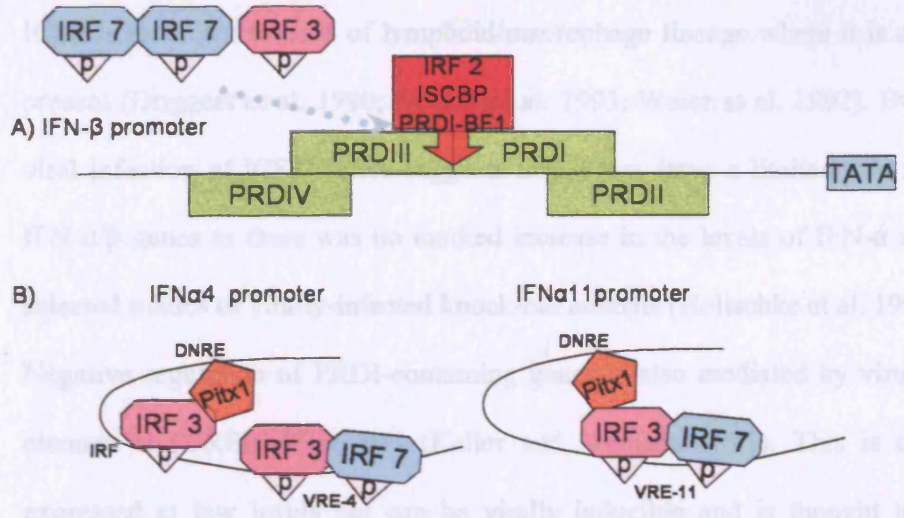

Figure 1.3 Regulatory elements of the IFN-β and IFN-A gene promoter

A) Interaction of regulatory activators with the human IFN-β gene promoter after virus induction. Transcription factors NF-$\kappa\beta$ binds to PRDII while IRF-1, IRF-3, IRF-7 bind to PRDI and PRDII domains on the human IFN-β promoter to upregulate IFN-β. However, these domains can also be occupied by repressors of the human IFN-β promoter e.g. IRF-2, ISCBP bind to PRDI and PRDIII, while PRDI-BF1 binds to PRDII, thereby preventing IRF-7 and IRF-3-mediated upregulation of IFN-β transcription (adapted from Doly et al, 1998). (All abbreviations are defined in the text).

B) Positive and negative regulation of type 1 IFN expression by Pitx1 is dependent upon the promoter elements on the IFN-α subtype gene. Pitx1 attaches to the DNRE region and also binds to an IRF(3 or 7) attached to the promoter region of the IFN-α gene to prevent the IRF complex from initiating transcription of the gene. However, attachment of Pitx1 to the DNRE does not repress the IFN-A4 promoter because here, the IFN-α4 promoter contains an additional IRF binding sequence in between the DNRE and the VRE, that enables IRF3 bound to Pitx1 to attach to, preventing Pitx1from interacting with IRF3 and IRF7 bound to the VRE-4 promoter sequence. In contrast, the IFN-α11 promoter sequence lacks the IRF binding site, so the only IRFs available for interaction with Pitx1 are IRF3 or IRF7 bound to the VRE-11 promoter and this therefore prevents expression of IFN-α11 (adapted from Island et al, 2002).

ICSP is restricted to cells of lymphoid/macrophage lineage where it is constitutively present (Driggers et al. 1990; Nelson et al. 1993; Weisz et al. 1992). Evidence from viral infection of $ICSP^{-/-}$ mice suggests that it may have a limited role in repressing IFN-α/β genes as there was no marked increase in the levels of IFN-α and IFN-β in selected tissues of virally-infected knock-out animals (Holtschke et al. 1996).

Negative regulation of PRDI-containing genes is also mediated by virus responsive element B (VREB)-PRDI-BFI (Keller and Maniatis 1991). This is constitutively expressed at low levels but can be virally inducible and is thought to be a post-induction repressor of the IFN-β gene.

IRF4 reduces type 1 IFN production by down regulating TLR signalling (Negishi et al. 2005). It competes with IRF5 for MyD88 and is probably part of a host response to control expression of pro-inflammatory genes, as IRF4 knock-out mice have high levels of pro-inflammatory cytokines and are hypersensitive to DNA-induced shock. IRF7 is associated with Epstein-Barr virus (EBV) latency and is also able to repress transcriptional activation by both IFN and IRF1 of an element containing an ISRE (Zhang and Pagano 2001).

Another target for repression of expression of ISGs is the transcription factor NF-κB, which is responsible for the activation of transcription of ISGs. NF-κB is activated by phosphorylation via the JAK/STAT pathway, and the gain of the phosphate moieties enables the protein to translocate into the cell nucleus to initiate transcription of ISGs. Kβ inhibitory proteins (IκB) modulate NFκB/Rel activity on up-regulation of IFN-β gene in response to viral infection (Beg and Baldwin 1993). IκB inhibits NF-κB activity by sequestering the protein in the cytoplasm and expression of IκB is also upregulated during viral infection, increasing the rate of sequestration.

The promoter region of certain IFN-α subtypes also contains specific cis-acting repressor elements which may contribute to differential expression of these α subtypes during viral infection. A distal negative regulatory element (DNRE), which was first discovered in the IFN-α11 gene subtype which is present in the genome but not expressed, has since been identified in the promoter region of IFN-α4. The DNRE sequence is considered a silencer region as it antagonizes the inducibility of the VRE promoter upstream of IFN genes. Silencing of IFN-α11 gene is mediated by the transcription factor PitX which has dual role as an activator or silencer of type 1 IFN gene expression. PitX blocks IFN-α11 induction by attaching to a DNRE region, associating with the translocated IRF3-IRF7 heterodimer and abolishing their transcriptional activity (Island et al. 2002) (Figure 1.3 B). While the DNRE element may be responsible for the permanent repression of the IFN-α11 gene, its role in IFN-α4 regulation is more complex as the presence of an IRF binding region close to the DNRE acts as a antisilencer to override the effect of bound Pitx1. IRF3 binds the IRF region, and Pitx1 associates with this IRF3 rather than IRF3 on the IRF3/IRF7 complex.

The presence or absence of silencer and anti-silencer elements could play a role in the differential expression of IFN-α genes. Expression of IFN-α subtypes after viral infection is also affected by variations in the sequence of the VRE in their respective promoter regions, which would affect how well the promoter is recognized by the corresponding DNA-binding factors or the related transcription activators (Civas et al. 2002).

1.4.5 Effector activities of type 1 IFNs

1.4.5.1 Direct antiviral effects of type 1 IFNs

IFNs-α,-β and -λ on their own do not posses any antiviral activity - they are important messengers that activate transcriptional of genes that encode direct antiviral activity. Type 1 IFNs released from infected tissue cells can help contain the spread of virus infection by upregulating expression of these proteins and creating an antiviral state in surrounding infected cells (Goodbourn et al. 2000; Le Page et al. 2000a; Mamane et al. 1999).

Type 1 IFNs activate the expression of several hundred IFN-stimulated genes (ISGs) (Der et al. 1998). A subset of the ISGs whose production is up regulated by type 1 IFNs have direct antiviral effects. Three IFN-activated antiviral pathways are particularly well characterised. These comprise the protein kinase PKR, activated by dsRNA, which suppresses cellular protein translation and promotes apoptosis; the enzyme 2'5'-OAS that produces short 2,5' oligoadenylates that activate RNAse L, which destroys viral and host RNA (Doly et al. 1998); and Mx proteins, which are GTPases that are necessary for control of orthomyxovirus infections (Arnheiter et al. 1996; Hefti et al. 1999; Pavlovic et al. 1999).

Other antiviral ISGs include ISG20, promyelocytic leukaemia protein (PML) and guanylate-binding protein 1 (GBP-1) (Anderson et al. 1999; Espert et al. 2003; Regad and Chelbi-Alix 2001; Taylor et al. 2000), as well as P56 and RNA-specific adenosine deaminase 1 (ADAR1) (Guo et al. 2000; Hui et al. 2003; Samuel 2001), two antiviral proteins reported to limit replication of HCV (Taylor et al. 2005). Additionally, NF-κB and ATF-c-Jun are activated in response to various stimuli in order to regulate the expression of genes relevant to inflammation.

1.4.5.2 Activating effects of type 1 IFN expression on the innate immune system

Type 1 IFNs have activating effects on many innate immune system cell types, thus indirectly contributing to antiviral effecter functions of the innate immune system and also helping to promote the induction of the adaptive immune response. IFN-α directly augments NK cytolytic function by inducing the lytic pathway, leading to secretion of perforin and granzyme B. IFNγ production is also up-regulated, augmenting development of Th1-type adaptive responses as well as further activation of the innate response, e.g. macrophage activation (Le Page et al. 2000a).

There is also evidence that type 1 IFNs upregulate gene expression of TLRs 1, 2, 3 and 7 in primary human macrophages after infection with influenza A and Sendai viruses (Miettinen et al. 2001)

DC maturation is directly triggered and promoted by type 1 IFNs. They stimulate migration of DCs into lymphoid tissues where they can interact with T cells. Simultaneously, they increase the expression of MHC class I on the surface of DCs, thus increasing antigen presentation, and also increase the expression of co-stimulatory molecules such as CD80, CD83, CD86 and CD40 that are important in DC:T cell communication (Biron 2001). Type 1 IFNs appear to preferentially induce maturation and functional expression of co-stimulatory molecules from myeloid $CD11c^+$ DCs over plasmacytoid $CD11c^-$ DCs (Ito et al. 2001), although they are required by plasmacytoid DCs for survival (Kadowaki 2000).

Type 1 IFNs have been reported to induce expression of various chemokines in DCs. *In vitro* generated monocyte-derived immature DCs, generated in the presence of GM-CSF plus IL-4 and then stimulated with type 1 IFNs upregulated CXCL9, CXCL11, and CXCL10 (the latter also upregulated in type 1 IFN stimulated DCs matured in GM-CSF + type 1 IFNs) (Padovan et al. 2002; Parlato et al. 2001). Each of

these chemokines binds the CXCR3 receptor expressed by activated T- and B-cells and by plasmacytoid pre-DCs (Cella et al. 1999a; Loetscher et al. 2001; Shields et al. 1999) indicating that DCs stimulated with type 1 IFNs may promote recruitment of these cells into specific regions of the lymphoid tissue or into peripheral sites of viral infection or inflammation. Additionally, DCs generated by culture in GM-CSF plus type 1 IFNs constitutively express CXCL19 and DC-CK-1 (Parlato et al. 2001), two chemokines involved in the recruitment of naïve T cells and therefore may be critical for initiation of immune responses (Adema et al. 1997). Type 1 IFN stimulated DCs also secrete IL-15, which in turn promotes DC maturation, suggesting type 1 IFNs may activate an autocrine stimulatory circuit involving IL-15 (Mattei et al. 2001).

1.4.5.3 Type 1 IFN as a modulator of the adaptive immune response

Type I IFNs are an important link between the innate and adaptive immune response (Kadowaki 2000). They not only help to activate DCs to become potent stimulators of T cell responses, but also by inducing cells of the innate immune system to produce cytokines such as IFN-γ and IL-12, they bias the response induced in a Th1 direction. Recent studies have shown that the use of type 1 IFNs as an adjuvant for soluble protein antigens results in stimulation of strong T cell responses, and that mice lacking the IFN-R respond poorly to complete Freund's adjuvant. This suggests a model for adjuvant responses where detection of viral/bacterial components (via TLR and other PRR) induces type 1 IFN production that primes DCs to become optimal antigen presenting cells which in turn activate and maintain Th1-type, CD8+ and CD4+ T cell responses and the production of antibodies of isotypes with good antiviral effecter functions (Le Bon et al. 2003; Le Bon et al. 2001; Le Bon and Tough 2002). Additionally, type 1 IFNs may also act directly on T or B cells to enhance

antigen-specific immune responses (Le Bon et al. 2006a; Le Bon et al. 2006b). During MCMV infection, production of low levels of IFN-β appears to be required for T cell survival (Banks et al. 2005).

Type 1 IFNs may also play an important role in the maintenance of memory T cell responses, by stimulating the production of cytokines such as IL-15 that drive low-level turnover of memory-phenotype T cells (Tough et al. 1996).

Type 1 IFNs also regulate the activity and/or expression of other cytokines ((Le Page et al. 2000b; Pestka et al. 2004; Theofilopoulos et al. 2005; Tough 2004). Basal expression of type 1 IFNs increases the signalling of IL-6 by providing docking sites for STAT1 and STAT3 on the IFNR1, which may bring it into close proximity with the gp130 chain of the IL-6 receptor (Mitani et al. 2001). The presence of type 1 IFNs also upregulates expression of transforming growth factor β (TGF-β), IL-1 receptor agonist and TNF (Brassard et al. 2002). Conversely, STAT6 activity is downregulated by IFN α/β through secretion of IL-4 which in turn induces expression of SOCS-1 (Dickensheets et al. 1999). IFN-α/β also antagonizes expression of another anti-viral cytokine, IL-12, although it upregulates transcription of the IL-12 receptor's β2 subunit in $CD4^+$ T cells (Biron 2001; Trinchieri 2003).

1.4.5.4 Systemic vs. local IFN induction and action

Most cells use the intracellular helicase pathway to detect viral infection and respond with type 1 IFN production. These are cells likely to be the first host cells infected upon initial exposure to the virus. Here, production of type 1 IFN is dependent upon activation of pre-existing IRF3 and upregulation of IRF7 (Kawai et al. 2005; Seth et al. 2005). It can be thus argued that IRF3 may be important in generating localized type 1 IFN reponses early in an infection as a means to control viral spread and clear

the infection. However, IRF7 is absolutely critical for control of systemic infection coupled with high viral load, as in this scenario, plasmacytoid DCs are major contributors to type 1 IFN production and the principal pathway for type 1 IFN induction in these plasmacytoid DCs is the TLR7/ TLR9-MyD88-TRAF6-IRF7 pathway and not intracellular IRF3-mediated induction.

In such a situation, activated plasmacytoid DCs migrate to T cell zones of secondary lymphoid organs to help prime naïve T cells (Colonna et al. 2004; Nakano et al. 2001). Thus the type 1 IFNs secreted by these plasmacytoid DCs may act locally to induce maturation of other DCs in the vicinity and ultimately aid the priming of $CD8^+$ T cell mediated adaptive immunity. It may be likely that systemic vs. local induction of type 1 IFN, which seeks to employ different IRFs for induction of type 1 IFN, represents a host-derived mechanism to discriminate between a mild viral infection, one which only affects a limited part of the body and can be controlled by local antiviral responses, from a more virulent infection needing a systemic response and T cell mediated clearance.

1.4.5.5 Negative regulatory effects of type 1 IFN

In addition to their immunostimulatory effects, type 1 IFNs may also have negative regulatory effects on their ability to modulate cell proliferation and survival. For example, as well as stimulating T cell responses, type 1 IFNs have an anti-proliferative effect on T cells. The mediated effect is thought to depend both on the levels of type 1 IFN present and the timing of the exposure of T cells to type 1 IFNs during the immune response. The anti-proliferative effects of type 1 IFNs may be critical to proper functioning of adaptive immunity. If left unchecked, excessive proliferation of T cells may lead to immunopathological damage. T cells become

refractory to IFN (by down regulating IFN-R) upon activation as a means of resisting apoptosis (Dondi et al. 2003). Studies on the effect of pre-existing levels of IFN-β on the functions of DCs found that IFN-β plays a dual role in shaping the T cell response, crucially, the timing of the exposure to IFN-β determined whether it promoted or inhibited generation of a functional T cell effector response (Nagai et al. 2003). When immature DCs were exposed to IFN-β and then matured, the matured DCs primed T cells which lacked IFN-γ production, and downregulated levels of TNF-α and LT, cytokines normally associated with a Th1 profile. Furthermore, pre-existing IFN-β reduced DC secretion of IL-12 which is needed for T cell expansion, and down-regulated expression of IL-18. Pre-existing IFN-β also elevated IL-10 levels in differentiated effector Th cells, thus dampening the cytotoxic immune response.

IFN-α/β is also able to exert inhibitory effects on B cell development and survival. Bone and spleen cellularity were found to be depleted in IFN-α treated mice and B-lineage cells were reduced by more than 80%, especially levels of $CD19^+$ pro-B cells (Lin et al. 1998a). Furthermore, type 1 IFNs may inhibit apoptosis of mature B cells. Hahmn *et al* (2005) also report that continuous expression of type 1 IFNs may suppress maturation of bone-marrow DC precursors.

1.5 Cellular components of the innate immune response

1.5.1 Dendritic cells

Dendritic cells were first described in 1973 by Steinman and Cohn (Steinman and Cohn 1973; Steinman et al. 1974; Steinman et al. 1980). They are sparsely distributed cells of hemopoietic origin. There are numerous types of DCs in the peripheral tissues such as the skin and in the blood and lymphoid tissues, the lineages between which are unclear. Phenotypic analysis has demonstrated that DCs are a highly

heterogeneous population. Many subtypes of DCs have been described with overlapping functions. In the spleen of mice, DC can be classified into three main subsets: $Ly6C^-$ $B220^-$ $CD8\alpha^-$ $CD11b^+$ $CD11c^+$, which may be further subdivided on the basis of $CD4^{+/-}$ expression (myeloid DCs), $Ly6C^-$ $B220^-$ $CD8\alpha^+$ $CD11b^-$ $CD11c^+$ (lymphoid DCs) and $Ly6C^-$ $B220^+$ $CD11b^-$ $CD11c^{lo}$ (plasmacytoid DC). The $CD11c^+$ lymphoid and myeloid DC subsets are together termed conventional DCs. Additionally, another subset of conventional DCs, termed interstitial DCs, have been isolated from the lymph node (Villadangos and Heath 2005), while yet another DC subset, defined by CD205 expression, is found only in the lymph nodes that drain the lungs, kidney and liver (Belz et al. 2004). Langerhans cells, which are present in the skin, comprise yet another DC subset.

Splenic conventional DC subsets do not appear to traffic through peripheral tissues before reaching the secondary lymphoid organs, but instead may be derived from precursors that traffic directly to the spleen and lymph nodes via the blood (Villadangos et al. 2001). The development pathway of plasmacytoid DCs remains controversial. It has been variously suggested that they are generated from a common myeloid precursor that is able develop into any DC subset (del Hoyo et al. 2002; Wilson et al. 2003), that plasmacytoid DCs are generated from a committed lymphoid precursor (Grouard et al. 1997) or that plasmacytoid DCs may be a subset of lymphoid cells undergoing programmed conversion into myeloid cells (Comeau et al. 2002; Zuniga et al. 2004). What has been established is the requirement for Flt3 ligand in the development process of DCs in general and specifically for plasmacytoid DC generation, as Flt3L k/o mice lack plasmacytoid DCs and posses reduced numbers of myeloid and lymphoid related ($CD8\alpha^+$) DCs (Brawand et al. 2002; D'Amico and Wu 2003; Karsunky et al. 2003; McKenna et al. 2000). Granulocyte-macrophage

stimulated factor (GM-CSF) may also be required as it enhances plasmacytoid DC migration from the bone marrow (Arpinati et al. 2000; Pulendran et al. 2000).

DCs in the spleen possess a high turnover rate, of about 2-5 days and even quicker upon activation (Kamath et al. 2000). As analysis of the blood did not reveal large influxes of replacement DCs or their precursors (O'Keeffe et al. 2003), it is likely that the source of replacement precursor cells are pre-DC precursors that seed the spleen and lymph nodes directly from the bone marrow (Villadangos and Heath 2005).

The frequency of DC belonging to each subtype may differ in different mouse strains. Using a plasmacytoid DC specific antibody, 120G8, Asselin-Paturel et al (Asselin-Paturel et al. 2003) analyzed the frequency of this population in the spleens of different strains of mice. They found that the frequency of $120G8^+$ plasmacytoid DCs was lowest in C57BL/6 mice (6.7% of total splenic DCs) and highest in 129Sv mice (27.8% of total splenic DCs). In contrast, the frequency of the splenic $CD8^+$ DCs subset among total DC was higher in C57BL/6 strains than in 129Sv (20.2% vs. 2.6%). Such differences could affect the ability of the specific strains to overcome infections by distinct pathogens.

1.5.1.2 Role of DCs in antiviral innate immunity

DCs are often the first cells of the immune system that responds to a pathogen due to both their location and their ability to sense the presence of a pathogen directly, e.g. via PRRs or by detecting the presence of cytokines released by other cell types in response to infection, e.g. the release of type 1 IFNs. All DCs have strong phagocytic ability, being able to internalise soluble proteins, microbes and pieces of dying cells from the extra cellular environment. They also sample the environment by macropinocytosis, forming large pinocytic vesicles in which extracellular fluid and

solutes are sampled (Sallusto et al. 1995). DCs also express receptors that mediate adsorptive endocytosis, including lectin receptors like the macrophage mannose receptor and DEC-205, as well as Fcγ and Fcε receptors (Sallusto et al. 1995);(Jiang et al. 1995). Direct recognition of the pathogen is accomplished through the expression of a variety of pattern recognition receptors outside and inside the cell. Each splenic DC subset expresses a unique combination of TLRs, as outlined in Table 1.2 overleaf:

Table 1.2 Types of TLRs expressed by different splenic DC subsets (adapted from (Edwards et al. 2003))

DC subset	Relative levels of TLR mRNAs expressed								
	1	2	3	4	5	6	7	8	9
$CD11c^{hi}Ly6C^{lo}B220^{-}CD4^{+}$	+++	+++	-/+	+	+++	+++	+++	+++	+++
$CD11c^{hi}Ly6C^{lo}B220^{-}CD8\alpha^{+}$	+++	+++	+	++	-/+	++	-	++	+++
$CD11c^{hi}Ly6C^{lo}B220^{-}CD4^{-}$ $CD8^{-}$	+++	+++	+	+	+++	++	++	++	++
$CD11c^{dim}Ly6C^{hi}B220^{+}$	+++	++	-	++	+	++	+++	+++	++

Upon encountering a non-self component/antigen, the previously quiescent DCs begin their activation or maturation process, characterised by migration out of their resident environment coupled with upregulation of the chemokine receptor CD62L, which allows entry into secondary lymphoid organs such as the lymph nodes and the spleen (Cella et al. 1999a; Nakano et al. 2001; Tough 2004).

Activation is accompanied by production of a range of cytokines tailored to combat the pathogen. During viral infections, production of type 1 IFN or IL-12 by DCs are key to the initiation of inflammatory, innate and adaptive responses (Akira and Hemmi 2003; Akira et al. 2001; Trinchieri 2003). Also, secretion of cytokines and chemokines at the site of infection serves to recruit more monocytes and DCs from the blood, thereby alerting and amplifying the innate response (Banchereau et al. 2000; Shortman and Liu 2002).

DCs possess direct anti-viral activity, via secretion of type 1 IFN leading to inducement of an anti-viral state. A subset of dendritic cells have been recently described that displays strong NK cell-like activity (Chan et al. 2006).

Cytokine production by pathogen-activated DCs also regulates the anti-viral activity of other innate cells, such as NK cell function. Human and murine plasmacytoid DCs secrete type 1 IFN and IL-15 which promote NK cell cytolytic activity and cytokine production and prolong their survival (Ferlazzo et al. 2003; Walzer et al. 2005a). During MCMV infection, interactions between murine NK cells and $CD11b^-$ conventional DCs have been shown to be integral to NK cell activation (Dalod et al. 2003). Engagement of the cell surface receptor NKG2D on NK cells and the NKG2D-ligand on the surface of MCMV-exposed $CD11b^+$ DCs provided the signals necessary to override the inhibitory signals provided by MHC I engagement to activate NK cytotoxic functions (Andoniou et al. 2005). Furthermore, NK cell capacity to produce

IFN-γ was reliant upon the polarized secretion of IL-12 and IL-18 released by the $CD11b^+$ DC subset. Finally, adoptive transfer of MCMV-activated $CD11b^+$ DCs resulted in improved control of MCMV infection, indicating that these cells participate in controlling viral replication *in vivo* (Andoniou et al. 2005). It should be noted that this NK-DC crosstalk is bi-directional - immature myeloid DCs physically interact with NK cells while the DCs supply NK cells with proliferative and survival signals such as type 1 IFNs and IL-15 (Moretta 2005), while cytokines such as TNF-α, IFN-γ and GM-CSF produced by NK cells can influence the progression of DC maturation.

1.5.1.3 Mechanism of type 1 IFN induction in splenic DCs

In many viral infections, DCs have been identified as the major type 1 IFN producer during early infection. The range of PRRs expressed on different DC subsets enables many components of the same virus to be detected, e.g. viral RNA/DNA by TLR 3/7/9 or RIG-I/MDA5 receptors, viral glycolipid by TLR 4 or DEC 205 and viral glycoproteins by TLR/lectin-type receptors (Haller et al. 2006). This, coupled with the means to signal for type 1 IFN via a number of adaptor molecules, such as MyD88, TBK or NF-κB, gives a level of redundancy in the system. This helps to ensure efficient type 1 IFN responses even if one component of the pathway is disrupted by the virus. Differential expression of TLRs on different DC subsets means that each subset is induced to produce type 1 IFN in response to a unique subset of stimuli. For example, plasmacytoid DCs are believed to have evolved to respond very rapidly to viral pathogens as they express TLR7 and TLR9 in the endosomal compartment (Beutler et al. 2006; Bowie and Haga 2005; Malmgaard et al. 2004) which serve to detect the presence of viral genomic components. Plasmacytoid DCs also possess the

intracellular RIG-I and MDA5 helicases but preferentially induce type 1 IFN via TLR signalling, a mechanism that probably evolved to allow swift and early production of type 1 IFN without the need for intracellular viral replication to occur in the plasmacytoid DCs. This means that plasmacytoid DCs can respond to the presence of infection themselves without becoming infected. As described earlier, plasmacytoid DCs are primed to produce high levels of IFN-α/β in the initial stages of infection to combat viral infections (Cella et al. 1999b; Colonna et al. 2002; Edwards et al. 2003; Siegal et al. 1999). Furthermore, plasmacytoid DCs consistently express lower levels of co-stimulatory molecules and are reported to be poor APCs compared to the other subsets, thus suggesting they may have evolved to be specialized type 1 IFN producing cells, rather than APCs (Asselin-Paturel et al. 2001; Grouard et al. 1997).

Conventional DCs preferentially express TLR3 and can sense viral and other dsRNA molecules in the endocytic compartment. They can thus respond to viral RNA from viruses with dsRNA genomes or dsRNA derived from uninfected cells provided they are taken up in the endosomal compartment (Schulz et al. 2005), again without the need to be infected themselves. However, conventional DCs can also produce type 1 IFNs in response to being infected. Experiments in which infected conventional DCs were infected with RNA viruses Sendai or VSV indicate that, in spite of differences in the route of entry, both viruses preferentially induced production of type 1 IFN via the helicase pathway and not the TLR3 activated signalling (Kato et al. 2005). The presence of TLR3 in conventional DCs may be predominantly used to recognize viral double-stranded RNA released from virally infected apoptotic cells sampled from the environment.

In summary, the combination of TLR expression and the capacity to sample the environment around them confers DCs (especially plasmacytoid DCs) with the ability

to initiate the first wave of type 1 IFN production even before they are intracellularly infected, unlike other cell types which can only respond to the presence of a virus when it enters the cytoplasm. This is a useful ability should the invading pathogen posses a means of blocking type 1 IFN induction in the cells it infects as this first wave of type 1 IFN secreted by the DCs can then activate antiviral genes and paracrine induction of type 1 IFN via the IFN-R JAK/STAT signalling pathway on the other cell types which, in the absence of DC-secreted type 1 IFN, would be susceptible to infection by the pathogen, and not be able mount a subsequent type 1 IFN-activated antiviral response if the infecting virus induced impairment of the type 1 IFN pathway.

1.5.1.4 Modulation of adaptive immunity by DCs

Perhaps the most important function of DCs is to transfer and translate cues from their surrounding microenvironment in order to inform and 'educate' the adaptive immune response. The ability to transfer an antigenic 'snapshot' of their environment is achieved via their antigen sampling and migratory capacities, which enable DCs to travel to lymphoid organs and expose circulating T cells to peripheral antigens that they would not have otherwise encountered. Additionally, DCs have the ability to translate environmental cues for the benefit of the host by possessing the capacity to activate different types of adaptive response or silence the adaptive immune response. As professional APCs, DCs are able to prime pathogen-specific adaptive responses and also tolerise the T cell response to self antigens. The specific role played by DCs (as activators or silencers) in host immunity depends upon the activation status, location as well as their cytokine expression profile. The range of cytokines secreted

is also subject to temporal constraints, namely different cytokine subsets are expressed during each stage of DC development and maturation.

In the absence of pathogens or a danger signal, DCs are present in tissues, lymphoid organs and the circulation in an immature, inactivated state (Henri et al. 2001; Wilson et al. 2003). In *in vitro* experiments, these immature DCs which express low levels of co-stimulatory molecules, do not interact productively with $CD8^+$ T cells (Albert et al. 2001). Unless DCs are in some way activated or licensed to produce immunity, the stimulation of T cells by DCs, will, by default, lead to tolerance. 'Inappropriately' activated DCs, for example, DCs induced to mature by TNF-α and prostaglandin E_2, are able to stimulate CD8 T cell proliferation but this leads to tolerance induction as the cells are eventually deleted. In order to be 'licensed', DCs must receive another signal, which can be delivered from CD4 T cells, via interaction of CD40L with CD40 on the mature DC cell surface (Cella et al. 1996; Schoenberger et al. 1998), although signals via type 1 IFN appear to overcome the requirement of CD4 T cell help (Le Bon et al. 2003). Licensing has been reported to require direct stimulation by pathogen associated stimuli (Sporri and Reis e Sousa 2005).

A possible mechanism for DC-mediated self tolerance *in vivo* has been put forward, based upon the observation that the vast majority of DCs in the spleen are in an immature state and thus express very low levels of co-stimulatory molecules; their role may be to establish peripheral tolerance during steady state while maintaining their capacity to respond to infections reaching the organs (Naik et al. 2006). Migration of tissue DCs into the draining lymph node occurs constitutively at a low level. These DCs have engulfed host cells and therefore present naïve T cells with self-antigens, but as they are in an immature state, the T cells are not active and so peripheral tolerance to self antigens is maintained. Therefore, mature but quiescent

DCs induce tolerance while activated, licensed mature DCs induce immunity (Reis 2006; Shortman and Heath 2001).

These biochemical signals, which determine the fate of naïve T cells, are referred to as Signal 1, 2 and 3.

1.5.1.4.1 Signal 1

Signal 1 refers to the physical interaction between pathogen peptide-MHC complexes expressed on the surface of the DC and antigen specific TCRs expressed by T cells (at the immunological synapse). This is believed to occur mainly in the lymphoid organs such as the spleen or the lymph nodes. Immature DCs, which are present in most tissues, possess several features that allow them to be specialized at antigen uptake. They are able to sample the antigens in their environment by phagocytosis and pinocytosis, forming large pinocytic vesicles in which extracellular fluid and solutes are sampled (Reis e Sousa et al 1993; Albert et al, 1998, Inaba et al, 1998). They also express receptors such as the mannose macrophage receptor (Engering et al. 1997; Sallusto et al. 1995), DEC-205 (Jiang et al 1995), Fcγ (Sallusto and Lanzavecchia 1994) and Fcε (Maurer and Stingl 1995) receptors that enhance adsorptive endocytosis. Unlike other cell types, captured antigen can be process by both the MHC class I (cytotoxic) and MHC class II (helper) pathways (Banchereau and Steinman 1998). These different mechanisms dramatically increase the amount of antigen available for presentation. Immature DCs also contain MHC class II rich compartments, to which exogenously-acquired antigens are targeted (Kleijmeer et al. 1995). After peripheral encounter with infection or antigen, DCs are induced to mature, reducing antigen uptake, increasing expression of MHC molecules on the cell surface and upregulating expression of co-stimulatory molecules and subsequently

migrate to lymphoid organs such as the spleen. This results in influx of matured DCs with high expression of the MHC class I and MHC class II molecules on the cell surface into the T cell zones of the lymph node and spleen (Itano et al. 2003; Lanzavecchia and Sallusto 2001). In mature DCs, MHC class II-peptide complexes are stably expressed on the surface for days providing a window for prolonged $CD4^+$ T cell stimulation (Cella et al. 1997a; Pierre et al. 1997; Winzler et al. 1997). Furthermore, the morphology of mature DCs alters to become cells with multiple long dendrites that can simultaneously engage multiple naïve CD4 and CD8 T cells (Miller et al. 2002).

All cell-cell interactions occur within defined structures of the spleen or the lymph node. Destruction of the lymphoid architecture in some cases disrupts adaptive immunity (Deonarain et al. 2003; Koch et al. 2005). $CD8^+$ T cell interactions with DCs occur in three successive stages: transient serial encounters during the first 8 hours in which the T cells decrease their motility and upregulate activation markers; formation of long-lasting stable T cell:DC conjugates and secretion of IL-2 and IFN-γ by T cells; and finally T cell migration at ~48hrs coinciding with onset of proliferation and reduction of DC contacts (Mempel et al. 2004).

1.5.1.4.2 Signal 2

Signal 2 refers to the accessory signals delivered to the T cell via co-stimulatory molecules e.g. through engagement of CD80 or CD86 on the cell surface of a licensed DC with CD28 on the T cell surface (Keir and Sharpe 2005) and CD40. DCs must be matured, via interactions with pathogen components, inflammatory mediators such as LPS, TNF-α or via $CD4^+$ T cell, in order for signal 2 to be delivered. In the absence of Signal 2, interaction between DC antigen-peptide MHC with naïve T cell TCR

(Signal 1) inactivates or tolerises the T cell by anergy, deletion or turning it into a regulatory cell. plasmacytoid DCs may also bring about tolerance via activation of regulatory CD4 T cells (Kawamura et al. 2006).

1.5.1.4.3 Signal 3

Signal 3 refers to signals from DC and other environmental sources to T cells that determine type of effector cell the T cell will become, for example differentiation into T helper 1 (T_H1) cells or T_H2 cells or cytotoxic T lymphocytes (CTL). Cytokines and other molecules secreted by various DC subsets define the milieu in which T cells proliferate. Expression of IL-12, IL-18 and IFN-α, which biases CD4 T cells towards a pro-inflammatory T_H1 cell fate, and production of Notch ligands which promote differentiation into T_H2 effecter cells, are examples of DC-mediated Signal 3.

It has been suggested that interaction with different DC subtypes may affect the outcome of the effector functions of the matured T cells. This proposal arises from the observation that each DC subset expresses a unique combination of TLRs and cytokine profile, which would then enable each subset to be optimised to promote distinct effector functions. For example, it has been reported that the $CD8\alpha^+$ and the $CD8\alpha^-$ DC subsets differentially prime Th1 vs. Th2 responses (Maldonado-Lopez et al. 1999; Pulendran et al. 1999). In addition, ony $CD8\alpha^+$ DCs are reported to be able to cross-prime $CD8^+$ T cell responses. Furthermore, treatment with LPS preferentially induced IL-12p70 production in the $CD8\alpha^+$ DC subset (Dillon et al. 2004; Pulendran et al. 2001), and finally the $CD8\alpha^-$ $CD4\alpha^+$ DC subset could not be induced to produce IL-12p70 in response to a range of innate stimuli (Agrawal et al. 2003; Manickasingham et al. 2003). However, while the subsets may be genetically programmed to differentially regulate the quality of the T cell response, these subsets

also exhibit a large degree of functional flexibility in response to environmental cues, as evidenced by reports that challenge the above hypothesis with observations that the same population of DCs could stimulate either Th1 or Th2 responses depending upon the stimuli it encountered (Boonstra et al. 2003; Huang et al. 2001; Vieira et al. 2000; Whelan et al. 2000).

In addition to educating and activating the cytotoxic T cell response, DCs have also been shown to modulate anti-viral B-cell development by secreting IL-6, which induces human B cells to differentiate into antibody-secreting plasma cells. Also, B-cells activated by plasmacytoid DCs preferentially secrete IgG instead of IgM, suggesting they may specifically target memory B cells (Jego et al. 2003).

1.5.1.5 Cross presentation

As mentioned above, DCs posses the ability to present exogenously acquired antigens on MHC class I molecules, a process termed cross-presentation (Heath et al, 2004). In most cell types, MHC class I molecules only present antigens present in the cytoplasm to ensure that only virally infected cells are recognized and destroyed by CTLs. Antigens from a variety of sources can be presented to $CD8^+$ T cells in this manner, but this process is especially important in generating CTL responses against targets that do not or can not infect professional antigen presenting cells such as DCs. These include immunological relevant antigens such as antigens acquired through uptake of apoptotic cells, virally-infected cells and tumour cells (Heath et al 2004). However, it appears that not all DCs are able to cross present, as recent reports suggest that cross-priming primarily occurs via the $CD8\alpha^+$ subset (Belz et al. 2005; Schnorrer et al. 2006) and may be promoted by activation of TLR3 (Schulz et al. 2005)

1.5.2 NK cells

NK cells play an important role in controlling many viral infections (Biron and Brossay 2001; French and Yokoyama 2003; Lodoen and Lanier 2006). They are cells of lymphoid origin that are present systemically, in the blood, spleen and lymphoid organs and in tissues (Yokoyama et al. 2004). NK cells are important in the control of tumour growth and viral infections, particularly in the control of herpesvirus infections, exerting their effects mainly through mediating lysis of malignant or virally infected cells and through production of antiviral cytokines such as IFN-γ (Biron et al. 1996; Kagi et al. 1994; Loh et al. 2005). In humans, NK cells constitute about 15% of peripheral blood lymphocytes. In mice NK cells constitute about 2.5% of splenic leukocytes and about 5-10% of peripheral blood. Typically, murine NK cells are defined by expression of CD122, NK1.1 and DX5 (Yokoyama et al. 2004)

Activation of NK cell effector functions is controlled by the balance of signalling through activating and inhibitory NK receptors (Lanier 2003; Moretta and Moretta 2004). The receptors are able to recognize normal cells of self origin through their surface expression of histocompatibility complex (MHC) molecules; in normal cells, activation of these receptors inhibits activity of NK cells, protecting the uninfected cell from lysis (Vivier et al. 2004). Virus infected or malignant cells often experience changes in protein expression, and often express MHC class I at reduced levels therefore they are less able to maintain suppression of NK cell activity, rendering them more susceptible to NK cell cytolysis (Cerwenka and Lanier 2001; Smyth et al. 2005). Triggering of NK cell lysis is also promoted by the interaction of activating NK cell receptors with ligands such as viral glycoproteins and stress-induced molecules on the surface of target cells.

NK cell activity is enhanced by the presence of cytokines such as IL-12, IL-15, IL-18, IL-21 and IFN-α/β (Nguyen et al. 2002a; Okamura et al. 1998; Orange and Biron 1996; Smyth et al. 2005). Activated NK cells are induced to secrete cytokines such as IFN-γ and TNF-α and are also able to mediate destruction of target cells by perforin-dependent binding of pathways and by death receptors such as Fas and TRAIL-R on target cells to induce apoptosis (Sato et al. 2001a; Smyth et al. 2001). Additionally, NK cells modulate other components of innate immunity by secretion of cytokines or signals that induce either maturation or apoptosis of dendritic cells and help to promote the development of Th1-type T cell responses (Andoniou et al. 2005; Della Chiesa et al. 2006; Gerosa et al. 2002; Gerosa et al. 2005)

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1.5.3 Macrophages

Macrophages are myeloid lineage cells with a wide tissue distribution. Monocytes which circulate in the blood differentiate into macrophages upon migration into tissues. These are phagocytic cells, which play an important role in controlling early bacterial and fungal infections. Macrophage mediate pathogen clearance by a variety of means – for example, they express pathogen recognition receptors such as the mannan receptor or CD14 that recognizes distinct sugars on bacterial cell surfaces and enable binding and killing of the bound pathogen (Aderem and Underhill 1999; Stahl and Gordon 1982; Tobias and Ulevitch 1993). Alternatively, macrophages are able to promote complement activation or phagocytosis that result in removal of the pathogen.

In addition to their role in the clearance of bacterial or fungal pathogens, macrophages may also help control viral infections, as studies in macrophage-deficient mice suggest they may be unable to clear viral infections (Karupiah et al. 1996; Seiler et al.

1997). Activated macrophages have been reported to produce IL-1, IL-6, IL-12 and TNF-α that enhance recruitment of lymphocytes to the site of infection as well as promote their functional activation (Salazar-Mather and Hokeness 2003).

1.6 Evasion of the innate immune system by viruses

1.6.1 Overview of viral immune evasion strategies

Many viruses have evolved strategies for interfering with host immune responses to promote their survival within the host. Such strategies may facilitate the duration of acute infections or permit the establishment and maintenance of chronic infections. These viral immunoevasion strategies may target both the innate and adaptive immune response, with innate cytokine responses DCs, NK cells, T cells and antiviral antibody responses all being evaded by different viruses (Ambagala et al. 2005; Andrews et al. 2005; Lodoen and Lanier 2006; Pollara et al. 2005). Immunoevasion strategies may be divided into tactics employed to avoid normal host immunosurveillance mechanisms or impair the host immune response. Tactics used to avoid normal host immunosurveillance include establishing latency, where, after the productive, acute phase of viral replication, virus remaining in the infected cell downregulates protein replication completely or to levels too low to be detected by the host response. Examples of viruses that can establish latency are HSV (latent in sensory neurons), varicella zoster virus (VZV) (latent in dorsal root ganglia) and Epstein-Barr virus (EBV) (latent in B cells). EBV is not fully latent in cells, as a single protein the Epstein-Barr virus nuclear antigen (EBNA)-1 is expressed to maintain the viral genome, but this protein is unable to elicit a T cell response. Human papilloma virus (HPV) is another virus that becomes latent, but during latency

expresses only low levels of viral proteins, thus avoiding detecting by the immune response (Frazer et al. 1999).

Another strategy employed by viruses is to infect cells in immunopriviledged sites, which are areas of little immune surveillance (Tortorella et al. 2000). Such areas include the central nervous system (CNS), the kidney, salivary glands and the anterior chamber of the eye. Examples of such viruses include HSV, VZV and measles virus which have tropism for the CNS, and are protected from CTL clearance low levels of MHC expression on cells of the CNS and by the blood brain barrier which limits trafficking of immune cells.

A number of viruses practice antigenic variation as a means to escape from host immune responses. Rhinoviruses and influenza viruses constantly alter the structure of epitopes of the dominant surface proteins haemagglutinin (HA) and neurominidase (N) such that strains quickly become unrecognizable by neutralizing antibodies generated by past infection with previous strains, permitting re-infection to occur. Such changes are termed antigenic drift (Tortorella et al. 2000). Antigenic variation is also employed to evade the CTL response. Mutation in viral CTL epitopes can abrogate epitope-specific responses by modifying peptide processing or transport, altering peptide binding to MHC class I or peptide interaction with the TCR, as discussed in more detail below. Antigenic variation is a strategy especially favoured by RNA viruses, including HCV, HIV, influenza and LCMV (in laboratory settings) in part because the proofreading mechanism of RNA polymerases is less efficient than DNA polymerase leading to higher error rates in the RNA genome (Elena and Sanjuan 2005). Thus each round of replication leads to an ever increasing number of genetic variants within the host, such that the virus exists as a quasi-species, and these variants are then subjected to selection pressure for fitness including the potential to

escape immunosurveillance. Other mechanisms of antigenic variation include reassortment and recombination of the viral genome (Hay et al. 2001).

Apart from avoiding the host's immune responses, certain viruses are able to impair immune functions in order to establish an infection. In the case of some viruses, infections may be associated with generalised immunosuppression; in many virus infections impairment occurs in a more restricted manner.

Measles virus is able to infect T cells, B cells, monocytes and DCs *in vitro*, and induce syncytium formation or apoptosis, therby destroying key immune cells (Joseph et al, 1975) with a knock-on effect on other immune responses as a result of reduced T and B cell function (reviewed in (Borrow and Oldstone 1995; Schneider-Schaulies et al. 2003) thus inducing a state of transient profound immunosuppression.

Dendritic cells are critical for both early innate responses and priming of T cells, and as such, activity of these cells is often subjected to impairment. Plasmacytoid DCs are often targeted as they act as viral sentinels and a source of type 1 IFN in peripheral sites of infection (Bautista et al. 2005; Freigang et al. 2005; Liu 2005; Rinaldo and Piazza 2004). There are several mechanisms by which viruses may interfere with DC function, but general strategies include alteration of the differentiation or signalling pathways, or modulation of DC effector functions in order to block induction of host T cell responses (Flano et al. 2005; Majumder et al. 2005; Walzer et al. 2005b)

NK dysregulation is another method of evading the innate immune system. Viruses, such as HCV and HIV that go on to generate a persistent infection, and even other viruses, like vaccinia which cause acute infections, have evolved means to modulate NK cell functions (Meier et al. 2005; Reading and Smith 2003). Viruses are able to interfere with either NK receptor-mediated recognition of virus infected cells by expressing homologs of MHC class I or modulating MHC expression on infected

cells, interfere with the release of NK-activating cytokines such as type 1 IFN, affect the intracellular activation pathway by antagonizing NK receptor functions and inhibit effector functions by blocking production of granzyme or Fas ligand (Dorner et al. 2004)

In T cells, multiple viruses are known to inhibit the MHC class I restricted antigen presentation pathway of infected cells to avoid recognition by $CD8^+$ T cells (Ambagala et al. 2005; Lilley and Ploegh 2005; Lybarger et al. 2005; Yewdell and Haeryfar 2005). HIV-1 impairs the T cell response by replicating in CD4+ T lymphocytes, rendering targets for destruction (Tortorella et al. 2000).

Also, several viruses are able to inhibit the presentation of lipids to CD1a-restricted T cells (Renukaradhya et al. 2005; Sanchez et al. 2005).

It is not uncommon for viruses to target more than one arm of the host immune response, or to employ multiple strategies for evading a given antiviral effector mechanism. For example, EBV escapes immunosurveillance by becoming latent in B cells, but the EBNA-1 protein which is expressed during latency is also able to avoid detection by $CD8^+$ T cell through interference of the antigen processing pathway to impede presentation by MHC class I molecules, reducing the surface MHC expression on infected cells.

HSV becomes latent in neuronal cells which are immunopriviledged because they express low levels of MHC class 1 molecules (Abendroth and Arvin 1999).

Herpes viruses and poxviruses express a variety of virally-encoded proteins which are soluble forms of immune receptors as a way to 'mop up' secreted cytokines and thus dampen the immune response, including soluble forms of TNF and IFN-receptors (Alejo et al. 2006; Couty and Gershengorn 2005; Nicholas 2005; Rosenkilde 2005; Sodhi et al. 2004). Certain viruses have evolved mechanisms to inhibit activation of

the apoptotic pathway in infected cells to reduce the availability of antigen for cross-presentation (Bowie et al. 2004; Boya et al. 2004; D'Agostino et al. 2005) or prevent cross-presentation by inhibiting phagocytosis (Cheung et al. 2005; Watts and Gommerman 2005).

Finally, many viruses also interfere with production of antiviral cytokine, type 1 IFNs, which is discussed in more detail in the next section.

1.6.2 Viral evasion of the type 1 IFN response

Many viruses have developed strategies to counteract the biological functions of type 1 IFN: these include viruses that cause both acute as well as persistent infections. Both DNA and RNA viruses have evolved means to down regulate the IFN-response. The multitude of strategies that viruses employ to evade this arm of the host immune response illustrates its importance in host defence. Interference mechanisms have been identified that cover the whole spectrum of the IFN response in infected cells.

The strategies employed may include blocking IFN induction or expression, preventing activation binding of IFN to the IFN-R by means virally encoded soluble receptors, blocking the intracellular signalling pathways involved in the IFN response directly down regulating transcription of ISGs with antiviral activity by interfering at the transcriptional level and possessing resistance to the antiviral effects of ISGs.

To evade type 1 IFN responses, some viruses use non-structural viral proteins that are otherwise non-essential for growth or replication. For example, while inactivated influenza viruses are good inducers of type 1 IFN, infection with live influenza virus inhibits the subsequent induction of IFNs by inactivated virus (Lindenmann 1960). Deletion of these non-essential genes results in type 1 IFN induction in the host and renders the virus susceptible to the consequent anti-viral effects (Ferko et al. 2004;

Talon et al. 2000b). Other viruses employ proteins that have additional functions in viral lifecycle to evade the type 1 IFN responses. For example, the phosphoprotein P constitutes an essential component of the viral polymerase complex in some RNA viruses. However, the P protein can have another role in dysregulating the IFN response. The P proteins of rabies, bornavirus and Ebola virus have been reported to inhibit activation of IRF3, mainly by interfering with TBK-1 mediated phosphorylation of IRF3 (Basler et al. 2003; Schlender et al. 2005; Unterstab et al. 2005).

Signalling via the PRRs is responsible for alerting the host to the presence of a viral pathogen and thus often presents a target for interference. The activation pathway may be disrupted by preventing recruitment of appropriate adaptor proteins or interfering with the phosphorylation of the adaptors (Boehme and Compton 2004; Bowie and Haga 2005; Finberg and Kurt-Jones 2004; Netea et al. 2004).

Viruses may prevent detection through host PRRs by concealing viral ligands that may potentially activate such receptors. For example, the NS1 protein of avian influenza prevents IFN induction by binding to and sequestering dsRNA (Fernandez-Sesma et al. 2006; Garcia-Sastre et al. 1998; Talon et al. 2000a; Wang et al. 2000)). Paramyxoviruses express V proteins that bind dsRNA and sequester them from the dsRNA-sensing helicase MDA5, preventing induction of type 1 IFN (Andrejeva et al. 2004)

The signalling pathway downstream of the PRR is also targeted. The NS3/4 A protein of HCV also interferes with the RIG-1 pathway (Foy et al. 2005) by enhancing degradation of membrane bound IPS1/MAVS complexes (Loo et al. 2006; Meylan et al. 2005).

Other viruses may block transcription of IFN-α/β by impairing the activity of the IRFs that activate transcription. Aside from the P proteins of rabies, bornavirus and Ebola virus mentioned above, the viral protein E3L expressed by vaccinia blocks phosphorylation of IRF 3 and prevents up-regulation of ISGs (Smith et al. 2001) and the NS3/4A serine protease expressed by HCV disrupts IRF3-dependent signalling by inducing cleavage of TRIF (Foy et al. 2003). Human herpes virus 8 (HHV-8), the causative agent of Kaposi sarcoma expresses homologues of IRFs (vIRFs) that either mimic the activity of cellular IRFs or exert a dominant-negative effect (Burysek et al. 1999a; Burysek et al. 1999b; Li et al. 1998; Lubyova et al. 2004; Lubyova and Pitha 2000; Zimring et al. 1998). A more global approach is applied by the NS protein of Rift valley virus, which prevents IFN production by inhibiting host cellular transcription (Billecocq et al. 2004; Le May et al. 2004).

Even when type 1 IFN is successfully produced, other strategies are applied that prevent the secreted cytokines from activating the IFN-R to initiate transcription of antiviral genes. Poxviruses posses a massive viral genome that encodes soluble IFN-binding proteins that compete with the IFN receptor for the secreted type 1 IFNs (Alcami et al. 2000; Symons et al. 1995). By 'mopping up' the type 1 IFNs produced, they permit viral spread by preventing induction of the autocrine IFN amplification loop and the establishment of an antiviral state in the surrounding uninfected cells.

The JAK/STAT signalling pathway is also targeted. Aside from sequestering viral nucleic acid, the V protein of paramyxoviruses also enhance proteosomal degradation of the IFN transcription factor STAT1 by promoting the ubiquitylation of the protein thus impairing JAK-STAT signalling pathway in the same cells (Didcock et al. 1999; Garcia-Sastre 2004; Precious et al. 2005; Ulane et al. 2003). The NS1 protein of the Rift Valley virus activates SOCS-1 which inhibits signalling by interacting with IFN-

R1 and may also block Tyk2 phosphorylation of STAT1 (Fenner et al. 2006; Haller et al. 2006). In order for this to be effective, SOCS-1 must be activated to engage IFNR1 very early in infection. Anti-sense segments of RNA encoding the NS proteins are packaged into infectious virus particles which can be immediately transcribed into NS mRNAs upon entry and uncoating in the cytoplasm, giving the virus a head start in blocking the JAK-STAT pathway (Ikegami et al. 2005). A similar approach is also adopted by HSV-1, which activates SOCS-3 to down regulate STAT1 and JAK phosphorylation (Yokota et al. 2004).

The consequence of activation of the type 1 IFN pathway is the production of a host of genes with antiviral functions. These genes are also targets for certain viruses. The NS1 gene product of influenza virus activates a host protein $P58^{IPK}$ which in turn inhibits the activity of PKR, reducing the activity of this antiviral protein. HSV secretes a product, infected cell protein 0 (ICP0) in the initial stages of infection which can both interfere with the JAK/STAT signalling pathway as well as directly down regulate transcription of ISGs. The virus also produces a PKR inhibitor, Us11. Viruses may also suppress IFN gene expression by global inhibition of host gene transcription (Ahmed et al. 2003; Billecocq et al. 2004; Fernandez-Sesma et al. 2006) (Thomas et al. 2004). Lytic viruses especially adopt this approach as it not only inhibits production of antiviral cytokines, but also expression of apoptotic factors and thus prolong the lifespan of the host and the number of progeny virions that can be produced from a single infection event. Diversion of host transcription machinery for the purpose of viral replication also enhances the rate of production of progeny virions. Poliovirus, vesicular stomatitis virus and bunyaviruses have been reported to adopt this strategy (Curiel et al. 1993; Fernandez-Sesma et al. 2006; Weber et al. 2002; Yalamanchili et al. 1997; Yuan et al. 1998).

Finally, as the IFN-response is the initial signal that sets off a cascade of events, virus-mediated inhibition of one component of the pathway will affect the regulation of distant signalling or effector molecules, thus interference at the early portion of the circuit amplifies the resulting inhibitory effects. So inhibition of the JAK-STAT pathway will suppress production of all IFN-inducible proteins, which not only include proteins with direct antiviral activity but also proteins essential for proper sensing of viral components such as RIG-1, MDA5 IPS-1/MAVS, IRF-3 and IRF-7 (Haller et al. 2006; Takaoka and Yanai 2006). Furthermore, the 'second-wave' of IFN production will be reduced, leading to contraction of the IFN response and promotion of viral infection and spread. These observations suggest that type 1 IFNs are a critical weapon in the immune response against these viruses that has helped to shape the evolution of these pathogens.

1.7 Immunity during persistent viral infections

There are instances where neither the innate nor the specific adaptive response is able to clear a virus infection completely. The virus is instead maintained in the host, and may persist for the lifetime of the host.

The levels of ongoing viral replication vary in different persistent infections. Some viruses remain latent for the lifetime of the host with occasional or sporadic reactivation, e.g. HSV-1, whilst others establish a latent infection with low levels of ongoing viral replication, as observed in cytomegalovirus (CMV) and EBV infections (reviewed in (Ahmed et al. 1997)). Alternatively, there may be high levels of ongoing viral replication. This requires that virus replication is sufficiently non-cytopathic to allow replication levels to be tolerated by the host, as is the case with hepatitis B virus

(HBV), HCV and LCMV infections, or that a renewable cell type is infected, as is the case with HIV and lactic dehydrogenase virus (LDHV).

Where there is constant (especially high level) viral replication, antigens and virally infected cells will constantly be present and encountered by the host immune system. The effect this has on the host innate response which is normally activated by the presence of infection, is not fully understood. Studies carried out in a variety of persistent infections have indicated that although innate responses may be highly activated in the acute phase of infection, this is a transient response that is not sustained over time. Instead, a much lower level of ongoing innate activation, or in some cases abnormalities in the innate response are observed in the context of chronic infection.

Analysis of the type 1 IFN response in the acute and chronic phases of infections with viruses that readily establish persistent infections has revealed a strong type 1 IFN response in acute infection that is not subsequently maintained (Abbate et al. 2003; Abel et al. 2002; Su et al. 2002; Thimme et al. 2002). A recent study of endogenous levels of IFNα/β and ISG mRNAs in the livers of chronic HCV patients found that the levels of type 1 IFNs were significantly downregulated in comparison to levels in non-infectious liver diseases (Abbate et al. 2003). The situation is similar in HBV infections where type 1 IFNs are produced at a lower level in chronic HBV infection (Bertoletti and Gehring 2006). The mechanisms involved are not well understood; although viral interference with IRF3 activation/functions has been implicated in impairment of IFN production by non-cytopathic bovine viral diarrhoea virus (BVDV) and HCV (Baigent et al. 2002; Foy et al. 2003).

In HIV infection, type 1 IFN is transiently upregulated in the serum in the acute phase of infection, but declines to undetectable levels prior to the peak in viral replication

(von Sydow et al. 1991). Again, the mechanisms involved are not well understood. The number of DCs present in peripheral blood is known to decreased from acute infection onwards (Pacanowski et al. 2001; Soumelis et al. 2001). PBMCs from HIV-infected individuals exhibit an impaired capacity to produce type 1 IFNs in response to *in vitro* stimulation with e.g. HSV, which is thought to reflect both the reduction in plasmacytoid DC frequency and an impairment in DC responsiveness.

DCs may become refractory to continuous activation by viral components during persistent infections, as is thought to occur during infection with malaria or HIV and in LPS-activated DCs which stop producing IL-12 after continuous stimulation with LPS (Cella et al. 1997b; Knight et al. 1991; Perry et al. 2005). Defects in DC functions may also be induced as a consequence of virus infection of these cells, e.g. DCs from chronic HIV and HCV patients have been reported to have a poor T cell stimulatory capacity and a population of DCs are thought to be infected with virus in each of these infections. Furthermore, depletion of DCs during infection may alter the DC-T cell ratio, leading to inappropriate activation of co-stimulatory molecules and therefore non-functional activated T cells.

As with DCs, abnormalities in the NK cell numbers, subset composition and/or functions have been observed in a number of human persistent infections (Goodier et al. 2003; Meier et al. 2005). Notably, although NK cell numbers are reduced during chronic HIV infection and the function of these cells is progressively impaired as infection progresses (thought to be due in part to alterations in the NK cell subset composition and in part to alterations in the relative levels of activating/inhibitory receptors on NK cells), the activity of NK cells is actually enhanced during initial stages of viral persistence (Meier et al. 2005). The mechanisms underlying this have not been elucidated. Defects in the innates response in chronic viral infections may

promote viral persistence both directly or indirectly due to secondary effects on adaptive responses (aspects of which are also impaired in many chronic viral infections).

1.8 Murine Lymphocytic Choriomeningitis Virus (LCMV) infection

1.8.1 LCMV

LCMV belongs to the Arenaviridae family, which consists of a single genus, the Arenavirus. 18 species of arenaviruses have been so far identified as belonging to the genus; these are listed in Table 1.3. The viruses can be further subdivided into two antigenic groups on the basis of serological assays, and as this division also correlates with their geographical distribution, the two groups are termed the Old World group and the New World group. LCMV is an Old World species and the prototype member of the Arenaviridae (Buchmeier et al. 2001).

Arenaviruses are zoonotic and cause chronic infections in rodent species and bats indigenous to Europe, Africa, the Americas and perhaps other continents. As the natural reservoir of the viruses, infected rodents often do not appear to display overt symptoms of disease. However, many of the viruses can be passed on to all other mammals including humans, (which are an incidental host in the virus' replicative cycle) usually through exposure to infected rodents and/or their excrement (CDC) 2005a). In humans, such infections may result in severe, even lethal hemorrhagic fevers or meningitis in the infected individual, as noted in the Table 1.3 ((CDC) 2005a; (CDC) 2005b). Some arenaviruses, such as Lassa and Machupo are subsequently able to be spread via human to human contact through direct exposure to the blood or excrement of infected patients or even via airborne transmission. Treatment with the antiviral drug ribavarin during the early stages of infection has shown some success in ameliorating disease ((CDC) 2005a; (CDC) 2004).

Table 1.3 Old World and New World arenaviruses (adapted from(Buchmeier et al. 2001))

Old World Arenavirus			
Virus	**Host in nature**	**Geographic distribution**	**Disease in humans**
Lymphocytic choriomeningtis (LCMV)	*Mus domesticus* *Mus musculus*	Europe, Americas	Lymphocytic choriomeningitis (aseptic meningitis)
Lassa Fever	*Mastomys species*	West Africa	Lassa fever
Ippy	*Arvicanthus* spp.	Central African Republic	-
Mopeia	*Mastomys natalensis*	Mozambique, Zimbabwe	-
Mobala	*Praomys*	Central African Republic	-
New World Arenavirus			
Junin	*Calomys musculinus*	Argentine pampas	Argentine hemorrhagic fever (AHF)
Machupo	*Calomys callosus*	Beni region of Bolivia	Bolivian hemorrhagic fever (BHF)
Guanarito	*Sigmodon alsotni, Zygodontomys brevicauda*	Venezuela	Venezuelan hemorrhagic fever (VHF)
Sabia	Unknown	Brazil	Brazilian hemorrhagic fever (VHF)
Whitewater Arroyo	*Neotoma albigula*	New Mexcico	Acute hemorrhagic fever
Tacaribe	Unknown (possibly bats)	Trinidad	-
Amapari	*Oryzomys gaeldi, Neacomys guianae*	Brazil	-
Parana	*Oryzomys buccinatus*	Paraguay	-
Tamiami	*Sigmodon hispidus*	United States	-
Pichinde	*Oryzomys albigulans*	Colombia	-
Latino	*Calomys callosus*	Bolivia	-
Flexel	*Oryzomys* spp	Brazil	-
Oliveros	*Bolomys obscurus*	Argentina	-

1.8.2 Characteristics of the LCMV virion and genome

The virus particle is lipid enveloped with a pleomorphic but normally spherical structure, with an average diameter of 90 to 110 nm. It is covered with surface glycoprotein spikes, enclosing the viral nucleoprotein complex and ribosomes acquired from host cells. The name Arenaviridae derives from the 'sandy' appearance of the virus particle (Burns and Buchmeier 1993). The viral genome consists of RNA in two segments of unequal length, totalling ~10.6 kb altogether. The genome of several strains of LCMV has been sequenced and the S or short RNA has been found to be between 3,366 to 3,535 nucleotides in length (Salvato 1989; 1993). The sequence of the long or L segment has been sequenced for LCMV as well as Tacaribe and Lassa fever viruses and ranges from 7,102 to 7,279 nucleotides. The genes on both the S and the L segments are arranged in an ambisense manner, encoding two proteins in opposite orientations, separated by an intergenic hairpin, as illustrated in Figure 1.4. The S RNA segment encodes the 63 kD nucleoprotein (NP) at the 3' end and the envelope glycoprotein precursor GP-C at the 5'end. GP-C is post-translationally cleaved by host proteases to yield two glycoprotein subunits GP-1 (44 kD) and GP-2 (35 kD). The L RNA segment also contains two genes, the gene for the 200 kD viral polymerase L protein is located on the 3' end while the 5' end contains the gene for the Z protein, a small protein (11 kD) with a RING-finger zinc binding domain (Cornu and de la Torre 2002).

The NP and polymerase genes at the 3' end of the segments are encoded in the conventional negative sense and are therefore expressed through transcription of genome-complementary mRNAs. The genes located at the 5' ends of the S and L segments, which encode the GP-C and Z proteins, are encoded in the mRNA sense, but they are only expressed through genomic sense mRNAs which are transcribed

from full length complementary copies of genomic RNAs that function as replicative intermediates. On both segments, the 3' end of the genes have been mapped to the intergenic region (IGR) which range from 59-217 nucleotides in length and is predicted to form one to three energetically stable, G-C rich, stem-loop (hairpin structures) in both the genomic and anti-genomic sense, which may play a role in stabilizing the 3' end of the mRNAs, suggesting structure-dependent transcription termination (Figure 1.4) (Borrow and Oldstone 1997). Recent studies have addressed the function of the IGR using minigenome analogues encoding reporter genes in an ambisense orientation with or without an intergenic region to separate the genes (Lee and de la Torre 2002). This work showed that the intergenic region did not affect the efficiency of anti-genome replication, but transcription of mRNAs of the reporter genes encoded at the 3'end of IGR-deficient minigenomes was hardly detectable, suggesting that the intergenic region plays a role in transcription termination for enhanced gene expression. Interestingly, this study also revealed a second role of the IGR in virus assembly and/or budding, which are required for the efficient propagation of LCMV infectivity (Pinschewer et al. 2005).

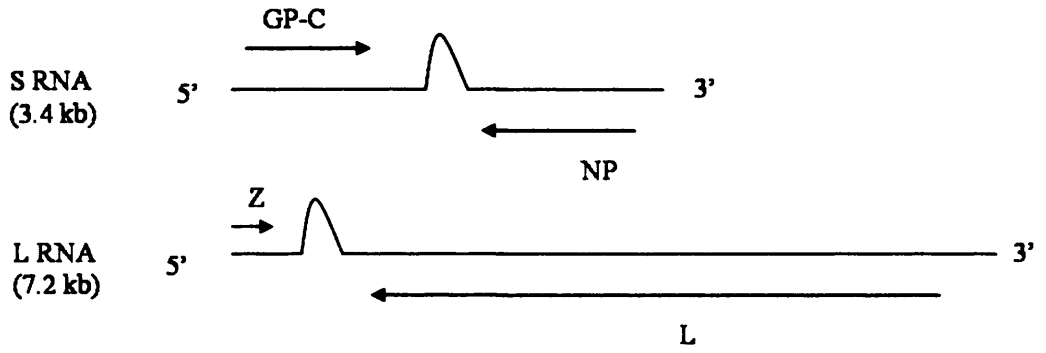


Figure 1.4 The genomic organisation of LCMV

The ambisense coding strategy of the LCMV RNA genome is outlined. GP-C, glycoprotein precursor NP, nucleoprotein; L, putative viral polymerase; Z, zinc-binding protein (from Borrow and Oldstone, 1997).

1.8.3 Viral proteins of LCMV

The external surface of the viral envelope is studded with glycoprotein spikes composed of glycoprotein subunits GP-1 and GP-2 which are derived from a precursor protein GP-C. GP-C contains a 58-amino acid signal peptide sequence that is cleaved from the amino terminus before GP-C is transported from the endoplasmic reticulum. The protein is glycosylated into GP-1 and GP-2 in the secretory pathway between the medial and trans-Golgi network. Cleavage occurs at or near a dibasic arginine-arginine motif with the amino-terminal portion of GP-C which becomes GP-1 and is mediated by the subtilase SKI-1/S1P (Beyer et al. 2003; Kunz et al. 2003). GP-1 contains four to seven potential N-linked glycosylation sites; it is heavily glycosylated, with evenly-distributed glycans accounting for about 35% of its total mass. GP-2 contains one to four potential N-linked glycosylation sites, all of which may not be occupied. GP-1 and GP-2 stay non-covalently attached until they arrive on the virion surface, where the subunits interact and assemble into the glycoprotein spike. It is hypothesized that GP-2 adopts the structure of an integral transmembrane 'stalk', with GP-1 forming a club-head spike on the external surface of the virion. It was long believed that GP-2 molecules assembled into homotetramers to form the stalk; however, recent studies have cast doubt on this hypothesis (Eschli et al. 2006). Cryomicroscopy studies clearly distinguished the stalk (presumably GP-2) from the spherical head region (presumed to be composed of GP-1). The spikes were visualized as single-lobed or double-lobed structures 9.5 nm in height and either 6.7 nm (single-lobed) or 7.9 nm (double-lobed) in width. Computer modelling alignment uncovered a high degree of similarity between GP-2 and other well characterised viral transmembrane proteins of the retrovirus and filovirus family, which consisted of trimeric subunits. Recent circular dichromism analysis and dynamic light scattering

experiments performed on GP-2 suggests that GP-2 forms a trimeric intergral membrane stalk. Each spike is built of three GP-1/GP-2 heterodimers, where GP-1 forms the globular head of the spike possibly with some linkage via disulfide bonding. In summary, this study concluded that GP was present on the virion surface as a trimeric protein, confirming its status as a member of the class I viral fusion protein family (Eschli et al. 2006).

The GP-1 subunit of the glycoprotein spike is the virion attachment protein that mediates interaction with receptor(s) on the host's cell surface (Kunz et al. 2003). This subunit is also the target for neutralizing antibodies that inhibit receptor binding interactions. Four highly variable surface-exposed regions thought to be in close proximity to the binding site have been characterized and are believed to aid in immunoevasion of neutralizing antibody responses by the virus. The viral glycoprotein not only mediates attachment to host cells, but also the fusion of virion and host cell membranes required for viral entry into host cells.

NP is the major structural protein of the virion. It is the major protein component of nucleocapsids and associates with virion RNA in the form of beadlike structures. NP is able to become phosphorylated in the later stages of acute infection and this form continues to be expressed during persistent infections *in vitro* (Fuller-Pace and Southern 1988; Oldstone and Buchmeier 1982). In infected cells, full-length NP is localized exclusively in the cytoplasm.

The L protein encodes for an RNA-dependent RNA polymerase. It has six characteristic sequence motifs conserved among other RNA polymerases of negative-strand RNA viruses (Buchmeier et al. 2001). It also contains unique regions conserved in the L proteins of ambisense viruses of the Arenavirus and Bunyavirus family. The polymerase has been reported to associate with the viral nucleopcapsids

in order to be active (Pinschewer et al. 2003). The active site of the polymerase was recently determined to be on domain III. A series of site-directed mutagenesis studies identified conserved residues within the domain that were strictly required for polymerase activity (Sanchez and de la Torre 2005).

The Z protein is the smallest protein encoded by the viral genome. It contains a RING-finger binding motif and is a zinc-binding protein. The exact function of Z is unclear, but it has been detected in both cytoplasm and the nucleus of LCMV-infected cells, suggesting it may play a role in DNA transcription/modulation, and it has also been found to promote budding of progeny arenavirus virions (Perez et al. 2003; Perez et al. 2004). Immunodepletion of Z protein in Tacaribe virus-infected cells strongly inhibited viral synthesis of genomic RNA and NP mRNA but it appears to be only required during the early stages of infection (Cornu and de la Torre 2002) (Jacamo et al. 2003; Lopez et al. 2001).

1.8.4 Viral entry, transcription and translation

Viral entry into host cells is mediated via binding of the virion surface glycoprotein GP-1 to host cell receptors, one of which is α-dystroglycan (Cao et al. 1998). α-dystroglycan is a highly conserved, peripheral membrane glycoprotein that is expressed in almost all cells. Correspondingly, LCMV has a broad *in vivo* tropism, infecting multiple tissues throughout the body of mice, although within individual tissues, particular cell types are preferentially targeted for infection (Fazakerley et al. 1991). Tropism for different cell types may thus depend on the levels of α-dystroglycan expressed (Borrow and Oldstone 1997).

While α-dystroglycan has been identified as the predominant attachment protein, the observation that α-dystroglycan-deleted cell lines could be infected with LCMV,

albeit with difficulty, indicates that there are other cellular receptors recognized by the virus (Kunz et al. 2004). LCMV isolates differ in their dependence on α-dsytroglycan for host cell entry, and correspondingly exhibit some differences in in vitro tropism and pathogenicity (Sevilla and de la Torre 2006).

The virion is taken into the cell by viropexis in large-smooth-walled endocytic vesicles. A fusion event between virion and endosomal membranes releases the contents of the virion into the cytoplasm, initiating the infection and replication process. GP-2 may play an integral role in membrane fusion and viral uncoating. LCMV fusion is dependent upon a drop in the pH. It is thought that the acidic pH environment of the endosome may destabilize the glycoprotein stalk, releasing the GP-1 subunit from the spike (Borrow and Oldstone 1994; Di Simone and Buchmeier 1995; Di Simone et al. 1994). This exposes a previously buried fusion peptide on the GP-2 stalk, which inserts into the host membrane. The GP-2 subunit is thought to undergo a series of conformational changes to give rise to a fusion pore, facilitating transfer of the viral nucleoprotein complex into the cytoplasm, where transcription takes place.

The RNA dependent RNA polymerase is thought to posses two distinct methods of transcription that generate RNA copies of the LCMV genome for different purposes. The transcriptase activity synthesizes capped (Meyer and Southern 1993; Raju et al. 1990) unencapsidated (Raju et al. 1990) mRNA that will be used as template for translation of viral proteins. These molecules posses 3' ends that are heterogenous, unpolyadenylated and end within the vicinity of the predicted hairpin in the IGR (Meyer and Southern 1994; Tortorici et al. 2001). The replicase function of the polymerase directs synthesis of mostly encapsidated (Raju et al. 1990), uncapped full-

length antigenomic and genomic RNA species, which are used as templates for further mRNA transcription and also packaged into new viral particles (Meyer et al. 2002).

Soluble NP protein is required for RNA replication in order to encapsidate nascent viral RNA during replication. Evidence for this comes from observations that full length genomic and antogenomic RNA species are found almost exclusively in encapsidated form (Raju et al. 1990) and arenavirus replication is dependent on ongoing protein synthesis and inhibited by translation inhibitor (Franze-Fernandez et al. 1987; Salvato et al. 1988), although the addition of exogenous NP restored viral replication in the presence of inhibitors (Salvato et al. 1988; Tortorici et al. 2001). Transcription and replication levels were also enhanced as levels of NP in the system increased, suggesting both processes were highly dependent on NP (Pinschewer et al. 2003).

NP mRNA and protein are the first viral components to accumulate in the infected cells (Fuller-Pace and Southern 1988). NP mRNA has been detected as early as 2 hours post-infection. The L protein can be detected at 12 to 24 hours after infection (Fuller-Pace and Southern 1989). Synthesis of GP-C and Z, which are encoded on the 5' ends of the genome segments, cannot begin until viral RNA replication starts and the full-length replicative intermediate viral-complementary RNAs of S and L is available as template for GP-C and Z mRNA transcription. The ambisense coding strategy of LCMV thus allows for differential expression of viral mRNAs, temporally separating expression of NP and L proteins from GP-C and Z expression.

Host cell enzymes are hijacked to send translated viral proteins to the Golgi for appropriate post-translational modifications (Wright et al. 1990). The glycosylated envelope glycoproteins are transported to the cell surface where virion assembly occurs. The assembly process is poorly understood. Newly synthesized genomic

RNAs, NP protein and L protein are assembled into nucleopcapsids which also incorporate host cell derived ribosomes (Fuller-Pace and Southern 1988; Salvato 1993). Progeny virions bud from the cell surface using glycoprotein-containing host cell membrane as their lipid envelope.

LCMV infection is typically non-cytopathic. Mice can tolerate a high level of LCMV replication without showing any major pathological effects (Borrow and Oldstone 1997) but nonetheless excrete high titres of infectious virus in their urine. For example, LCMV-infected guinea pigs may excrete up to 10^5 pfu/ml of virus in the urine. In fact, exposure to excreted LCMV in mouse or hamster urine, cage dust or litter is a source of laboratory-acquired LCMV infection (Hinman et al. 1975).

The L polymerase is an RNA-dependent RNA polymerase with low-fidelity. As such, the LCMV genome is subject to high rate of mutation. Within an infected host, LCMV thus exists as a heterogeneous population or quasispecies that continually generates viral variants (Dockter et al. 1996; Sevilla and de la Torre 2006). Under selection pressure, for example in certain tissues of organs of the host, viral mutants may emerge that have acquired key advantagous mutations. For example, mice that have been infected with LCMV Arm from birth often posses variants in the liver and lymphoid tissue that contain a mutation that changes amino acid at position 260 in the GP-1 subunit from phenylalanine to leucine (Evans et al. 1994; Salvato et al. 1991; Villarete et al. 1994). It is believed that this mutation confers on the virus an enhanced ability to infect macrophages and dendritic cells as well as other related cells such as Kupffer cells in the liver (King et al. 1990; Matloubian et al. 1993; Matloubian et al. 1990). By contrast, the original strain of LCMV with the phenylalanine at position 260 predominates in other organs such as the heart and central nervous system. Numerous isolates of LCMV have been isolated in such a manner.

1.8.5 Outcome of LCMV infection of mice

LCMV is a natural pathogen of mice, although it can also infect other mammalian species including humans. In mice, LCMV can cause either an acute infection or a persistent infection in which the virus may be present for life (Borrow and Oldstone 1997). A number of isolates of LCMV have been characterized, which differ in their in vivo pathogenicity. The outcome of an infection depends on both host and viral factors, including the age, strain and immunocompetence of the mice infected; and the particular LCMV isolate used and dose and route of infection.

Infection of adult immunocompetent mice with a low/moderate dose of most LCMV isolates via a peripheral route results in an acute infection, followed by clearance of the virus in 10-14 days. Clearance is primarily achieved through perforin-mediated lysis of virus-infected cells by CD8+ T cells (Kagi et al. 1994; Leist et al. 1987b; Moskophidis et al. 1987b; Walsh et al. 1994b).

However, if the same dose of virus is delivered via the intra-cranial (i.c.) route, there is viral replication in the meninges and choroid plexus resulting in targeting of the CTL response to these sites. This causes lethal meningitis, which is induced by CTL-mediated inflammation and not by viral replication itself (Cole et al. 1972; Gilden et al. 1972a; Gilden et al. 1972b).

If mice become infected with LCMV *in utero* or within 48 hours of birth, LCMV-specific T cells are clonally deleted in the thymus as self tolerance is established (Pircher et al. 1989). In the absence of an effective LCMV-specific CD8+ T cell response, the mice are unable to clear the infection and life-long persistence results. In nature, LCMV is maintained by persistent infection of specific species of rodent hosts (such as *mus musculus* and *mus domesticus*).

LCMV is also able to establish a persistent infection in adult mice if they are infected under circumstances where viral replication is able to reach high titres in the initial stages of infection. This can be achieved by delivery of high doses of virus through the systemic i.v. route; by using fast replicating and IFN-resistant LCMV strains such as Armstrong Clone 13, WE Docile and Traub; and by infection of immunocompromised hosts, such as knock-out mice lacking the type 1 IFN-R (Borrow and Oldstone 1997). If there is high level viral replication as the virus specific CD8+ T cell response is induced, T cell exhaustion occurs, i.e. virus specific T cells are either deleted or rendered non-functional (Moskophidis and Lehmann-Grube 1983; 1984; Zajac et al. 1998). The mechanism(s) responsible for CTL exhaustion are unclear, but it may reflect 'overstimulation' of CD8+ T cells during chronic LCMV infection causing them to undergo massive expansion (Fuller and Zajac 2003; Gallimore et al. 1998; King and Goodbourn 1998; Wherry et al. 2003a), which may drive them to terminally differentiate and undergo apoptic death. Wherry *et al.* (2003) have proposed that in chronic LCMV infections, activated CD8+ T cells undergo distinct stages of CTL 'exhaustion', with sequential loss of cytokine production leading to deletion or death. This may be a means to control immunopathology to the host. A strong correlation between viral load and effector function was also observed whereby T cells to highly displayed and/or persisting epitopes were exhausted more quickly. The presence of CD4+ T cells helped delay the exhaustion period (Wherry et al. 2003a). Another mechanism suggested to contribute to CTL exhaustion is inappropriate antigen presentation e.g. antigen presentation by non-professional APCS which may prime CD8+ T cells for anergy or deletion (Althage et al. 1992).

Barber *et al* recently described a novel pathway that may be involved in T cell exhaustion (Barber et al. 2006). The receptor programmed death (PD)-1 was found to

be greatly upregulated on exhausted LCMV-specific $CD8^+$ T cells (but not functionally competent cells) in persistently-infected mice; furthermore, PD-L1 (also called B7-H1), one of the ligands for PD-1, was expressed at very high levels on splenocytes from persistently-infected mice, especially on virus-infected cells. Blockade of the B7-H1-PD-1 pathway by a monoclonal antibody restored $CD8^+$ T cell function and reduced viral burden. The authors suggested that the PD-1/PD-L1 axis operated under conditions of sustained high levels of antigenic stimulation to reduce T cell responsiveness and that the PD-1 inhibitory pathway may have evolved to regulate immune-mediated damage during a persistent infection by turning 'off' the virus-specific T cells.

1.8.6 The host response during acute and persistent LCMV infection

1.8.6.1 Acute LCMV infection

Acute LCMV infection of mice, such as infection with a low dose of LCMV Arm delivered i.p. into immunocompetent adult mice, results in stimulation of both innate and effector pathways, resulting in clearance of the virus in 7 to 10 days.

1.8.6.1.1 Type 1 IFNs and LCMV

Like infection with most viruses, infection with LCMV very rapidly stimulates production of type 1 IFNs. Type 1 IFN mRNA is upregulated in the spleen within 24 hours of infection (Malmgaard et al. 2002) and levels of type 1 IFN in the serum peak at ~2-3 days post-infection (Jacobson et al. 1981). The mechanism of type 1 IFN induction by LCMV is still unclear but probably involves activation of the anti-viral TLR and/or the intracellular helicase pathways. Analysis of type 1 IFN induction in mice lacking IFN-R or STAT-1 production indicated that contrary to other viruses,

LCMV is a poor direct inducer of type 1 IFN and instead relies on autocrine amplification of type 1 IFN induction through the positive feedback loop involving signalling via the IFN-R and STAT-1-mediated transcription, to generate the high levels of type 1 IFN observed in serum of infected mice (Malmgaard et al. 2002). The predominant cellular source of the serum IFN in LCMV-infected mice are also unclear. Whilst plasmacytoid DCs are induced to produce type 1 IFNs very rapidly after LCMV infection (Montoya et al. 2005), depletion studies have suggested that unlike in other virus infections, they may not constitute the major source of type 1 IFNs (Dalod et al. 2002). However, an alternative principal IFN-producing cell type in LCMV-infected mice has yet to be identified.

Studies carried out using neutralizing antibodies against type 1 IFNs or type 1 IFN knock-out mice have shown that type 1 IFNs make a critical contribution to controlling the replication of LCMV, in particular, that of strains such as Arm and WE Aggressive (Moskophidis et al. 1994; Muller et al. 1992). Type1 IFNs likely act to combat LCMV infection via a combination of direct effects on viral replication and by modulating cellular immunity to ensure the induction of appropriate anti-LCMV CD8 T cell effector responses (Ou et al. 2001). Evidence for this is provided by the observations that strains that are resistant to the effects of type 1 IFN such as LCMV Cl13, Docile and Traub are the strains capable of establishing a persistent infection *in vivo* (Moskophidis et al. 1994) (illustrating the importance of antiviral effects of type 1 IFNs); but in addition, persistence of these (and other) LCMV isolates is promoted in mice treated with neutralizing antibodies against IFN-α/β or IFN-receptor knock-out mice (Moskophidis et al. 1994; Ou et al. 2001) (illustrating the importance of indirect, immunostimulatory effects of type 1 IFNs in control of LCMV infection).

1.8.6.1.2 Dendritic cells

DCs are infected by LCMV, with different strains/isolates of LCMV possessing varying degrees of tropism for the cells. In general, it has been observed that strains or isolates of LCMV that go on to establish a persistent infection preferentially infect DC subsets displaying $CD11c^+$ or DEC 205^+ expression, with reports suggesting that more than 80% of these splenic DCs become infected within the first 20 days by such strains. In contrast, infection with a strain of LCMV that is cleared found that the virus infected <10% of DCs (Borrow et al. 1995; Sevilla et al. 2000). It has therefore been postulated that the increased capacity to infect splenic DCs may be a factor that enables certain strains/isolates of LCMV to persist in the host.

Following acute infection with LCMV Arm, dendritic cells from all subsets are rapidly activated. As mentioned above, type 1 IFN production is initiated by plasmacytoid DCs within 24 hours of infection. Conventional DC subsets upregulate expression of MHC class I and class II and adhesion and co-stimulatory molecules such as CD40, CD80, CD86, and ICAM-1, produce proinflammatory cytokines, and acquire the ability to stimulate LCMV-specific $CD8^+$ T cells (Montoya et al. 2005). Endogenous type 1 IFN upregulation was shown to contribute to DC phenotypic activation. However, DC functional activation is transient; is rapidly downregulated in all DC subsets, and the numbers of $CD11c^{high}$ $CD8^+$ and $CD4^+$ DCs in the spleen declines as activated cells undergo apoptosis.

1.8.6.1.3 Natural killer cells

NK cells are activated in early LCMV infection as type 1 IFN are produced (Biron et al. 1983; Bukowski et al. 1983a; Orange and Biron 1996; Pien and Biron 2000). However, experiments carried out using beige mice (which have reduced NK cell

activity) or in which NK cells were depleted with antibodies to asialo-GM1 indicate that NK cells do not make a significant contribution to control of LCMV infection *in vivo*. This may be due at least in part to the fact that LCMV infection is not associated with activation of high levels of production of IL-12, a cytokine which is required to stimulate IFN-γ production in NK cells.

1.8.6.1.4 Antiviral humoral response

Antibody responses against LCMV are also generated early in infection, as early as 4 days post-infection; however, neutralizing antibodies (mainly directed against the glycoprotein) are only detected 20-60 days post-infection (Hotchin et al. 1969).The reasons for this are not well understood but may be related to depletion of B cells and B cell precursors by LCMV. B cell numbers were reported to fall during an acute LCMV infection and did not return to normal levels until at least 3 weeks post infection (Borrow et al. 2005). The virally-induced suppression was found to be dependent on the presence of TNF-α secreted in response to the viral infection.

Antibodies are not required for clearance of an acute LCMV infection, although they do contribute to control of viral replication and can help to prevent/clear persistent LCMV infections (Battegay et al. 1993). Importantly, the antibody response induced following clearance of an acute LCMV infection also provides protection against re-infection on subsequent viral exposure (Baldridge and Buchmeier 1992).

1.8.6.1.5 T-lymphocyte Response

T-lymphocytes, specifically the $CD8\alpha^+$ T cell population are absolutely necessary for clearance of an acute LCMV infection (Assmann-Wischer et al. 1985; Borrow 1997; Fung-Leung et al. 1991; Lehmann-Grube et al. 1988) and are able to mediate viral

clearance (and therefore effector functions) in the absence of $CD4^+$ T cells or expression of MHC Class II (Christensen et al. 1994; Kasaian et al. 1991). However, CD4 T cells do contribute to the resolution LCMV infection, as in the absence of $CD4^+$ T cells, infection with doses of LCMV that would cause an acute infection in immunocompetent adult mice resulted in establishment of a persistent infection (Matloubian et al. 1994). $CD4^+$ T cells can combat LCMV replication directly, e.g. via secretion of antiviral cytokines such as IFN-γ; and also play an important role in providing help for the generation and maintenance of $CD8^+$ T cell responses.

The kinetics of virus-specific $CD8^+$ T cell responses correlate well with clearance of acute LCMV infection. Effector cells are initially detected at day 4-5 post-infection and peak between days 7 and 9 post-infection, which coincides with resolution of the infection (Moskophidis et al. 1987a). In the first week after LCMV infection, active T cell expansion occurs and there are large increases in the total number of T cells in the spleen. By day 8 post-infection, the frequency of LCMV-specific $CD8^+$ T cells in the spleen is ~1 in 2 (Murali-Krishna et al. 1998). Expansion is followed by a contraction phase with large-scale apoptotic cell death that peaks at day 11 post-infection, after the virus is cleared (Razvi and Welsh 1993). The LCMV-specific T cells left are maintained as long-lived memory T cells which confer immunity against subsequent LCMV infections (Jamieson and Ahmed 1989; Lau et al. 1994; Whitmire et al. 1998).

$CD8^+$ T cells can combat LCMV infection by either cell contact-dependent lysis of virus-infected cells or by production of antiviral cytokines (which does not destroy the virally-infected cell). Evidence suggests that clearance by contact-dependent lysis is critically important for clearance of an acute infection. In perforin-deficient mice, infection with an acute strain of LCMV could not be cleared despite proper T cell activation (Clark et al. 1995; Kagi et al. 1994; Walsh et al. 1994a). By contrast, mice

with non-functional Fas controlled acute LCMV infection with normal kinetics indicating that Fas-dependent pathways were probably not critical (Kagi et al. 1994), although there is evidence of synergism between FasL and perforin (Rode et al. 2004). However, while perforin is critical for clearance of an acute LCMV infection, IFN-γ and TNF-α cytokines secreted by effecter T cells are required to control a persistent infection where the number of infected cells is much higher (Borrow 1997; Guidotti et al. 1999; Suresh et al. 2004).

1.8.6.2 Persistent LCMV infection

As described earlier, mice become persistently infected with LCMV either if they are infected with virus at birth, or if they are subsequently infected under conditions where virus replicates to high titres in the early stages of infection, e.g. following inoculation of mice with a high titre of an IFN-resistant strain of LCMV such as LCMV Cl13 or LCMV Docile.

In both situations, persistence is associated with a lack of $CD8^+$ T cell control of viral replication, but the mechanisms responsible for this differ. When a persistent infection is induced by infection of newborn mice, LCMV-specific T cells are clonally deleted in the thymus by mechanisms that give rise to tolerization, as the virus is recognized as self by the host (Pircher et al. 1991; Pircher et al. 1989). An LCMV-specific T cell response is thus not activated. The immune response of these persistently infected mice is otherwise functional, and specific T and B cell responses to other antigens are not impaired (Tishon and Oldstone 1987). By contrast, when a persistent infection is induced in adult immunocompetent mice by infection with high dose of certain isolates of LCMV, a virus-specific $CD8^+$T cell response is induced but it is then exhausted by functional impairment and physical deletion of LCMV-specific effector

$CD8^+$ T cells (van der Most et al. 2003; Wherry et al. 2003b). There is also evidence that LCMV Cl13 promotes persistence by subverting the innate arm of immunity.

1.8.7 Immunoevasion strategies of LCMV

As outlined above, the principal mechanisms by which LCMV avoids control of the host immune response involve failure to induce a virus-specific $CD8^+$ T cell response (as occurs in mice infected at birth with LCMV) and impairment of the function of virus-specific T cells (as occurs in wild type adult mice infected with a high dose of a persistent LCMV strain/isolate).

Additionally, LCMV may also apply other immunoevasion strategies to resist clearance by the host. LCMV isolates have been shown to vary in their susceptibility to control by type 1 IFNs, both in vivo and in vitro (Leist et al. 1987a; Moskophidis et al. 1994; Ou et al. 2001). Notably, those LCMV isolates that exhibit the greatest type 1 IFN resistance are those that are able to establish persistent infections on high dose inoculation into adult immunocompetent mice, suggesting that this IFN resistance may play a key role *in vivo*. LCMV consists of only 4 viral proteins and viral RNA, so at a glance it appears its capacity to interfere with the host's immune system is limited. However, 2 of the 4 proteins encoded, NP and Z, have the potential to interfere with host transcription factors. For example, NP is reported to interfere with the Pit1 transcription factor that regulates growth hormone production in mice infected at birth with LCMV (de la Torre and Oldstone 1992). Likewise, NP/Z may also be able to modulate the host's antiviral responses to confer a survival advantage upon the virus. Notably, the Z protein interferes with the actions of PML, an ISG, by sequestering the protein and preventing it translocating into the nucleus (Borden et al. 1998).

LCMV persistence may also be promoted by impairment of DC numbers and/or DC function. LCMV Cl13 which is able to cause a persistent infection in adult mice is thought to infect DCs more efficiently than LCMV Arm, a closely-related virus isolate that is more readily controlled by the host (Borrow et al. 1991; Salvato et al. 1991; Sevilla et al. 2000). Infection of DCs may target them for destruction by the host $CD8^+$ T cell response (Borrow et al. 1995; Odermatt et al. 1991) leading to impairment of their functions. Sevilla *et al* (2004) report that splenic DCs from mice with a long-term LCMV Cl13 infection exhibited reduced surface expression of CD40, CD80, CD86 and MHC class I and MHC class II. The total number of splenic DCs in persistently infected mice is also reduced (Sevilla et al. 2004), and Hahm *et al* (2005) postulated that the continuous presence of type 1 IFN in the system had deleterious effects on DC maturation and expansion, suppressing maturation of precursor cells in the bone marrow through a STAT2-dependent pathway (Hahm et al. 2005). Together, these abnormalities in splenic DC in mice persistently infected as adults with LCMV may account for observations that these animals (unlike mice persistently infected at birth) exhibit an impaired capacity to mount immune responses to unrelated antigens/pathogens (Tishon et al. 1993; Odermatt et al. 1991).

In addition to inducing tolerance or exhaustion of LCMV-specific $CD8^+$ T cells, LCMV may potentially also evade the T cell response. When mice expressing a TCR specific for LCMV were persistently infected with LCMV, viral variants able to escape recognition by the transgenic TCR expressing CTL appeared and were selected for within the viral quasispecies, resulting in failure of viral clearance (Aebischer et al. 1991; Pircher et al. 1990; Puglielli et al. 2001). However, this has only been observed in an artifical system using transgenic mice and it is unclear whether the virus adopts this strategy to remain in a wild-type host.

LCMV also possesses strategies for resisting control by the humoural response. In acute infection, the production of neutralizing antibodies is relatively delayed (Buchmeier et al. 1980; Cerny et al 1988; Bruns et al, 1983; Brundler et al 1996; Hangartner 2006) which may help to promote viral persistence in the host. The mechanisms involved are not clear but may include LCMV infection of B cells producing virus neutralizing antibodies, targeting these cells for destruction by the $CD8^+$ T cell response (Battegay et al. 1993; Moskophidis et al. 1992; Planz et al. 1996) and/or infection associated B cell heamatopoeises (Borrow et al. 2005). Most neutralizing antibodies to LCMV are directed towards the GP-1 surface protein. During persistent infection, GP-1 expression is downregulated in infected cells, possibly as a measure to avoid recognition by the host's antibody response (Oldstone and Buchmeier 1982).

1.8.8 Activation state of the innate system in mice persistently infected with LCMV

A persistent LCMV infection is productive, bathing the host with high concentrations of viral components. In mice persistently infected with LCMV, there is widespread high-level viral replication with continuous production of virus particles. All the stimuli to drive activation of the innate responses are thus present. Is the innate response correspondingly in a highly activated state?

Early studies that measured type 1 IFN activity during persistent viral infections reported different outcomes. Most of the work was conducted on mice which were infected at birth, and became persistently infected as a result of delection of LCMV-specific T cells. Studies by Bukowski *et al* (Bukowski et al. 1983b) on 6 month old congenitally infected mice reported them to have mid-elevated levels of plasma IFN,

where they were 4 times above baseline values, but still lower than IFN levels from acute LCMV mice. Merrigen *et a*l (Merigan et al. 1977) detected continuous production of IFN (not distinguished between -α, -β or -γ) in nude (*nu/nu*) athymic mice persistently infected with LCMV. Some groups reported low levels of IFN-α/β and the IFN-stimulated anti-viral molecule 2'5'OAS in the serum of persistently infected mice which, when induced with polyIC or NDV, produced undetectable or reduced levels of serum IFN compared to LCMV-uninfected mice (Holtermann and Havel 1970; Bukowski et al 1983; Saron et al. 1982). Conversely, Holtermann and Havell (Holtermann and Havell 1970) reported that congenitally infected mice did not produce IFN unless they were infected with an unrelated virus. Even then, IFN levels were 4-6 fold lower compared to those in non-LCMV infected mice.

Increased NK cytotoxic activity (compared to that of NK cells from uninfected mice), was also detected in persistently-infected mice (Bukowski et al. 1983a), which the authors attributed to sustained IFN-mediated stimulation of NK cells. The cytotoxic activity was intermediate between the low activity of uninfected mice and the high activity of acutely infected mice.

Mice persistently infected with LCMV have also been reported to have a reduced susceptibility to infection with certain viruses including equine encephalomyelitis virus, Rauscher leukemia virus and polyoma virus (as commented upon in (Bukowski et al. 1983b) and (Holtermann and Havell 1970)). Whether this is due to the elevated activation status of the innate system is unclear.

A recent study by Homann *et al* (2004) reported activation of DCs and virus-infected T cells in mice persistently infected *in utero* or at birth (Homann et al. 2004). These infected cells were also more prone to apoptosis. When infected with an second virus, the T-cell response was functional, although the total number of responding T cells

were reduced, suggesting that persistent LCMV infection from birth did alter the functions of the host's heterologeous immunity.

1.9 Objectives of the research project

Many viruses are able to infect and then persist in their hosts indefinitely. The effect such an infection has on the host's immune response, especially on the innate immune response, is still unclear. Firstly, if such viruses impair aspects of the innate responses in order to persist in the host, which of the response are affected, and how is this impairment mediated?

Secondly, what effect does constant exposure to virus and/or viral components have on the host's innate immune system – is it perpetually activate, or are responses downregulated.

Thirdly, how does the presence of persisting virus affect the ability of the innate immune systemt to response to other virus infections?

These are questions that have yet to be properly understood in chronic infections such as HIV, HBV or HCV. I planned to use the murine model of persistent LCMV infection to address some of these questions. Several factors make LCMV a good model virus to study aspects of persistent viral infections. Firstly, LCMV is a natural pathogen of mice, making it possible to study infection in a host in which the virus is adapted for persistence. Secondly, murine models are amenable to kinetic analysis and experimental manipulation. Thirdly, different strains/isolates of LCMV are available that cause either acute or persistent infection in mice. Also, unlike other viruses that become latent during persistency, persistent infection with LCMV is accompanied by high levels of systemic infection, as is observed in human infection with HIV, HCV or HBV. In addition, viral mechanisms can also be studied *in vitro* as

LCMV readily infects multiple cell lines and is able to establish a non-lytic, persistent infection in these cells. Finally, many reagents, e.g. LCMV-specific antibodies, and expression plasmids were available in the lab.

The objectives of my project were as follows:

1. To develop assays (i) to enable quantitation of low levels of bioactive type 1 IFN (using reporter gene systems) and (ii) to detect, quantify and distinguish between various subtypes of type 1 IFN at the mRNA level.

2. To employ the sensitive and specific assays developed above to follow the induction of type 1 IFN in serum and spleen during the early and late stages of a persistent LCMV infection *in vivo*, comparing the magnitude and kinetics of the response with that observed in an acute LCMV infection.

3. To determine whether there are impairments in type 1 IFN induction during chronic LCMV infection by investigating the responsiveness of persistently infected mice to TLR stimuli and infection with an unrelated RNA virus.

4. To assess the effects of LCMV infection on the ability of cell lines to produce type 1 IFNs in response to different *in vitro* stimuli.

5. To dissect the role of individual LCMV proteins in impairment of type 1 IFN induction using cell lines stably transfected with GP, NP or Z.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plasticware and other consumables

Product	Catalogue No.	Supplier
12-well Flat bottom Plates	3513	Corning Costar, High Wycombe, UK
13 mm Coverslips	ML-579-13	Western Lab Suppliers, Aldershot, UK
140 mm Nunclon TM Dishes	168381A	Life Technologies, Paisley, UK
15 ml Falcon Tubes	352096	Becton Dickinson Labware, NJ, USA
48-well Plates	150687	Life Technologies, Paisley, UK
50 ml Falcon Tubes	352070	Becton Dickinson Labware, NJ, USA
6-well Plates	353046	Becton Dickinson Labware, NJ, USA
96-well microtitre plate(Luciferase assay)	3917	Corning Costar, High Wycombe, UK
96-well V bottom Plates	3894	Corning Costar, High Wycombe, UK
Bijou Tubes	275/0460/24	BDH Lab Supplies, Poole, UK
Cell Strainers 40 μm	352340	Becton Dickinson Labware, NJ, USA
Cluster Tubes (Fluorescence activated cell sorter (FACS) tubes)	AB-0672	Abgene, Surrey, UK
Eppendorf Tubes 0.5 ml	96.4625.9.01	Tref Ag, Degersheim, Switzerland
Eppendorf Tubes 1.5 ml	96.8160.9.02	Tref Ag, Degersheim, Switzerland

Product	Catalogue No.	Supplier
FACS Tubes 5 ml	352054	Becton Dickinson Labware, NJ, USA
Finntips	9401-255	Life Sciences, Basingstoke, UK
Flasks T150	90151	Helena Biosciences, Sunderland, UK
Flasks T25	9026	Helena Biosciences, Sunderland, UK
Immulon-II Enzyme-linked immunosorbant assay (ELISA) Plates	3455	Dynex technologies, Middlesex, UK
Immuno 96-well Nunc ELISA Plates (Maxisorp)	439454A	Life Technologies, Paisley, UK
LS+ Magnetic activation cell sorter (MACS) Columns	130-042-401	Miltenyi Biotech, Bisely, UK
Microcon YM100	42412	Millipore Corp, Bedford, USA
Microscope Slides	406-0184-04	BDH Lab Supplies, Poole, UK
MS+ MACS Columns	130-042-201	Miltenyi Biotech, Bisely, UK
Needle, 18G 1.2x40 mm	1100-250016	Sherwood Medical, Northern Ireland, BT53 7AP
Needle, 19G 1.1 x 40 mm	197	Terumo Corp,
Needle, 27G,0.4x20mm	Z192384-100EA	Terumo Corp
Nunc Cryovial External Thread 1.8 ml	375418	Life Technologies, Paisley, UK
Nunc Cryovials Internal Thread 1.8 ml	377267	Life Technologies, Paisley, UK

Product	Catalogue No.	Supplier
Pasteur Pipettes	475-068	
Petri dish, 100x150 mm (BM-DC dish)	35-1029	Becton Dickinson Labware, NJ, USA
Petri dish, 35x10mm (BM-DC dish)	351008	Becton Dickinson Labware, NJ, USA
Pipettes 10 ml	47110	Bibby Sterilin, Stone, UK
Pipettes 25 ml	18327	Bibby Sterilin, Stone, UK
Pipettes 5 ml	40105	Bibby Sterilin, Stone, UK
Plate Sealers	EC/005.SPN	Camlab Ltd, Cambridge, UK
Slide-A-Lyzer (1000 MWCO) Dialysis Cassettes	6638122	Pierce & Warriner UK Ltd., Chester, UK
Standard Repette Dispenser Tips, 2.5 ml	488-007	Jencons Scientific, Leighton Buzzard, UK
Sterile Repette Dispenser Tips, 5 ml	488-008	Jencons Scientific, Leighton Buzzard, UK
Syringes 1, 2, 25, 50 ml	x	IAH Stores, Compton, UK
Tips 1000µl	GPS1000	Rainin Instruments Co Ltd., Woburn, USA
Tips 20µl	GPS25	Rainin Instruments Co Ltd, Woburn, USA
Tips 200µl	GPS250	Rainin Instruments Co Ltd., Woburn, USA
Universal Tubes	275/0460/04	BDH Lab Supplies, Poole, UK

Product	Catalogue No.	Supplier
Ribonuclease (RNase)-free 0.5ml microfuge tubes	12350	Ambion Europe Ltd, Huntingdon, Cambridgeshire, UK
RNase-free 1.5ml microfuge tubes	12450	Ambion Europe Ltd, Huntingdon, Cambridgeshire, UK
30µl pre-sterilized filter tips	RT-20F	Rainin Instruments Co Ltd, Woburn, USA
1000µl pre-sterilized filter tips	RT-1000F	Rainin Instruments Co Ltd, Woburn, USA
200µl pre-sterilized filter tips	RT-200F	Rainin Instruments Co Ltd, Woburn, USA
96-well flat-bottomed plates	3595	Corning Costar, High Wycombe, UK
96-well U-bottomed plates	3799	Corning Costar, High Wycombe, UK
Lab-Tek II Chamber slide, sterile, 8 chambers	154534	Nalge Nunc International, Naperville, IL, USA
Cell scrapers	353086	Becton Dickinson Labware, NJ, USA
Glasstic®Slide 10 combination coverslip microscope slides	87144	Hycor Biomedical, Garden Grove, CA, USA
Millex 0.22µl syringe filter	SLGP033RS	Millipore, Cork, Ireland.
Eppendorf Combipure 2.5ml dispensers	0030.069.447	Eppendorf, Hamburg, Germany
96-well polymerase chain	AB0900	Abgene, Surrey, UK

Product	Catalogue No.	Supplier
reaction (PCR) plate		
Domed cap strip for PCR plate	AB0852	Abgene, Surrey, UK
FACS tube with cell strainer, 5ml	352235	Becton Dickinson Labware, NJ, USA
Poly-L-lysine coated slides	J2800AMNZ	Menzel GmbH, Braunschweig, Germany
Teflon-coated glass slides	10-3114	Cel-line Associates Inc, Newfield, NJ, USA
Coverglass (22x50mm)	406/0188/44	BDH Lab Supplies, Poole, UK
Homogenization tubes 2ml	19201	Qiagen, Crawley, UK
Stainless steel cone balls, 6.0 mm diameter	22.455.0003C	Retsch (U.K.) Ltd, Leeds, UK
Tissue moulds	M497-12	Simport, Fisher Scientific UK, Loughborough, UK

2.1.2 Tissue Culture Reagents

Product	Catalogue No.	Supplier
2x Medium199	21157-029	GibcoBRL, Paisley, UK
Accutase	L11-007	PAA laboratories, Linz, Austria
Agar:Agar	1.01613	Merck, Darmstadt, Germany
Baby hamster kidney (BHK) Medium		GibcoBRL, Paisley, UK
Blasticidin S Hydrochloride	150477	ICN Biomedicals GmbH, Eschwege, Germany
Collagenase Type III	LS004182	Worthington Biochemical Corp. Reading, UK
Dubecco's modified Eagles' medium (DMEM) with Glutamax Medium	31955-021	GibcoBRL, Paisley, UK
Effectene Transfection reagent	301427	Qiagen Ltd, Crawley, UK
FACS Lysing Solution 10x	349202	Becton Dickinson Labwear, UJ, USA
Foetal Calf Serum (FCS)	A15-002	PAA laboratories, Linz, Austria
FuGENE 6 Transfection Reagent	1 815 091	Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN, USA
GeneJuice Transfection Reagent	70967-3	CN Biosciences, Beeston, UK

Product	Catalogue No.	Supplier
Good Foetal Calf Serum (GFCS)	10106-169	Invitrogen, Paisley, UK
Iscove's Modified Dulbecco's Medium (IMDM) with Glutamax	31980-022	GibcoBRL, Paisley, UK
Low Endotoxin Foetal Bovine Serum (FBS, Lot: 62K3376)	F9666	Sigma-Aldrich, Poole, UK
Modified Eagles medium (MEM) with Glutamax	41090-028	GibcoBRL, Paisley, UK
NycoPrep 1.077 A	1113029	Axis-shield PoC AS, Oslo, Norway
Opti-MEM I Reduced Serum Medium	51985-042	GibcoBRL, Paisley, UK
Penicillin and Streptomycin	x	IAH Media Supplies, Compton, UK
Polymyxin B sulfate salt	P4932	Sigma-Aldrich, Poole, UK
RPMI with Glutamax	72400-.021	GibcoBRL, Paisley, UK
RPMI with Glutamax	72400-.021	GibcoBRL, Paisley, UK
Saline	F7124	Baxter Healthcare, Compton
Trypan blue	15250-061	GibcoBRL, Paisley, UK
Trypsin-EDTA	45300-019	GibcoBRL, Paisley, UK

2.1.3 Chemicals and molecular biology reagents

Product	Catalogue No.	Supplier
100 bp Deoxyribonucleic acid (DNA) Ladder	G210A	Promega, Southampton, UK
1kb DNA Ladder	G571A	Promega, Southampton, UK
250 bp DNA Ladder	10596-013	Invitrogen Life Technologies, Paisley, UK
6-carboxyfluorescein (FAM)- 6-carboxy-N,N,N',N',-tetramethylrhodamine (TAMRA) dual-labelled oligonucleotide probes	-	Sigma-Genosys, Haverhill, UK , or Eurogentec, Seraing, Belgium
Acetone	32201	Sigma Aldrich, Poole, UK
Agarose LE Analytical Grade	A9539	Sigma Aldrich, Poole, UK
Ammonium Persulphate	161-0700	BioRad Laboratories Ltd, Hemel Hempstead, UK
Ampicillin	x	IAH Media Supplies, Compton, UK
Bgl II		Invitrogen Life Technologies, Paisley, UK
Blue/Orange 6x Loading Dye	G190A	Promega, Southampton, UK
Bovine Serum Albumin (BSA)	A7030	Sigma Aldrich, Poole, UK
Chloamphenicol acetyl transferase (CAT) ELISA kit	1 363 727	Roche Diagnostics Roche Molecular Biochemicals Mannheim, Germany

Product	Catalogue No.	Supplier
CpG1826		MWG, Ebersberg, Germany
Crystal violet	C3886	Sigma Aldrich, Poole, UK
Cytochrome 5 (Cy5) Ab Labelling kit	PA35000	Amersham Biosciences, Little Chalfont, UK
Cytokine Bead Array (CBA) Mouse Inflammation kit	552364	BD Biosciences, PharMingen, Oxford, UK
Deoxyribonuclease I, Amplification Grade	18068-015	Invitrogen Life Technologies, Paisley, UK
DH5α competent cells	18265-017	Invitrogen Life Technologies, Paisley, UK
Dimethyl sulphoxide (DMSO)	D2650	Sigma Aldrich, Poole, UK
DNAse I	AMPD1	Sigma Aldrich, Poole, UK
EcoRI	15202-013	Invitrogen Life Technologies, Paisley, UK
Endofree Plasmid Maxi Kit	12362	Qiagen Ltd, Crawley, UK
Ethanol	x	IAH Stores, Compton, UK
Ethanol, absolute, molecular biology grade	E7023	Sigma Aldrich, Poole, UK
Ethidium Bromide	E1510	Sigma Aldrich, Poole, UK
Ethylenediaminetetraacetic acid (EDTA)	100935V	BDH Lab Supplies, Poole, UK
FAM- Major groove binder (MGB) dual-labelled	-	Applied Biosystems, Warrington, UK

Product	Catalogue No.	Supplier
oligonucleotide probes		
Fluorescent mounting medium	S3023	DakoCytomation, Glostrup, Denmark
Formaldehyde	47629	Sigma Aldrich, Poole, UK
Glycerol	101186M	BDH Lab Supplies, Poole, UK
Halothane	-	IAH Supplies, Compton
HEPES buffer	15630-049	GibcoBRL, Paisley, UK
HindIII	15207-012	Invitrogen Life Technologies, Paisley, UK
Hydrochloric acid	30721	Sigma Aldrich, Poole, UK
ImmEdge wax pen	H400	Vector Laboratories, Peterborough, UK
Isopropanol	59080	Sigma Aldrich, Poole, UK
Lipopolysaccharide (LPS) *E.coli* 055.135	L2880	Sigma Aldrich, Poole, UK
Luciferase Assay System	E1500	Promega, Southampton, UK
Luria Broth (LB)	x	IAH Media Supplies, Compton, UK
Luria Broth Agar Plates	x	IAH Media Supplies, Compton, UK
Methanol	10158BG	BDH Lab Supplies, Poole, UK
Mouse IFN-α ELISA kit	42100-1	PBL Biomedical Laboratories, 131 Ethel Rd West Suite 6, Piscataway,

Product	Catalogue No.	Supplier
		NJ 08854, USA
Mouse IFN-α/β	Gift from Dr. Agnes Le Bon	Le Bon *et al.*, 2001
Nuclease-free water	9937	Ambion Europe Ltd, Huntingdon, Cambridgeshire, UK
OCT Compound	36160 3E	BDH Laboratory Supplies, Poole, UK
One Shot INVαF' Competent Cells	C2020-03	Invitrogen Life Technologies, Paisley, UK
One step revese transcriptase (RT) quantitative PCR (qPCR) Mastermix	RT-QPRT-032X	Eurogentec, Seraing, Belgium
OPD Tablets	P9187	Sigma Aldrich, Poole, UK
Paraformaldehyde	2944704L	BDH Lab Supplies, Poole, UK
Paraformaldehyde	P6148	Sigma Aldrich, Poole, UK
Phosphate Buffer Saline x10	14200-067	GibcoBRL, Paisley, UK
Polyinosinic acid:Polycytidylic acid, sodium salt, double stranded (Poly IC)	P0913	Sigma Aldrich, Poole, UK
QIAfilter Plasmid Maxi Kit	12263	Qiagen, Crawley, UK
Qiaprep Miniprep Kit	27104	Qiagen Ltd, Crawley, UK
Qiashredder	79654	Qiagen Ltd, Crawley, UK
R848	Tlrl-r848	AutogenBioclear, Calne, Wiltshire,

Product	Catalogue No.	Supplier
		UK
Recombinant Mouse granulocyte-macrophage colony stimulating factor (GM-CSF)	415-ML	R&D Systems, Abingdon, UK
Recombinant murine IFN-α	HC1040b	HyCult Biotechnology b.v., Uden, The Netherlands
Recombinant murine IFN-β	HC1050b	HyCult Biotechnology b.v., Uden, The Netherlands
RNase-Free DNase Set	79254	Qiagen Ltd, Crawley, UK
RNaseZap	9780-9782	Ambion Europe Ltd, Huntingdon, Cambridgeshire, UK
RNeasy Mini Kit	74104	Qiagen Ltd, Crawley, UK
Sequence detection oligonucleotide primers	-	MWG, Ebersberg, Germany
Sodium acetate (anhydrous)	S2889	Sigma Aldrich, Poole, UK
Sodium Azide	S-8032	Sigma Aldrich, Poole, UK
Sodium Carbonate Anhydrous	102405Y	BDH Lab Supplies, Poole, UK
Sodium Chloride	102415K	BDH Lab Supplies, Poole, UK
Sodium Dodecyl Sulphate (SDS)	444464T	BDH Lab Supplies, Poole, UK
Sodium Hydrogen Carbonate	102475W	BDH Lab Supplies, Poole, UK
Sodium Hydroxide	30620	Sigma Aldrich, Poole, UK
Sulphuric Acid	102761C	BDH Lab Supplies, Poole, UK
Taqman Universal PCR	4324018	Applied Biosystems, Warrington,

Product	Catalogue No.	Supplier
Mastermix		UK
Tris-acetate EDTA (TAE) 25x	0796-1.6L	Amresco, Ohio, USA
Tris-hydrochloric acid (Tris-HCL)	T3253	Sigma Aldrich, Poole, UK
Triton-X-100	T9284	Sigma Aldrich, Poole, UK
Trizma Base	T1503	Sigma Aldrich, Poole, UK
Tween-20	P7949	Sigma Aldrich, Poole, UK
B-mercaptoethanol	M7522	Sigma Aldrich, Poole, UK

2.1.4 Anti-murine antibodies for FACS analysis

Antibody to	Clone	Isotype	Conjugated to	Working dilution	Catalogue no.	Supplier
CD11c	HL-3	Armenian Hamster IgG1, λ	APC[1]	1 in 100	553802	BD Biosciences PharMingen, Oxford, UK
CD11c (N418)			MicroBeads		130-052-001	Miltenyi Biotec, Bisley, UK
CD19	ID3	Rat IgG2a, κ	Biotin	1 in 100	553784	BD Biosciences PharMingen, Oxford, UK
CD3e	145-2C11	Armenian Hamster IgG1, κ	Biotin	1 in 100	553059	BD Biosciences PharMingen, Oxford, UK
CD4 (L3T4)	RM4-5	Rat IgG2α, κ	FITC[2]	1 in 100	553046	BD Biosciences PharMingen, Oxford, UK
CD40	3/23	Rat IgG2a, κ	RPE[3]	1 in 100	553791	BD Biosciences PharMingen, Oxford, UK
CD49b/Pan-NK cells	DX5	Rat IgM, κ	Biotin	1 in 100	553856	BD Biosciences PharMingen, Oxford, UK

[1] APC = Allophycocyanin
[2] FITC = Fluorescein isothiocyanate
[3] RPE = Red phycoerythrin

Antibody to	**Clone**	**Isotype**	**Conjugated to**	**Working dilution**	**Catalogue no.**	**Supplier**
CD54 (adhesion molecule, ICAM-1)	3E2	Armenian Hamster IgG1κ	RPE	1 in 100	553253	BD Biosciences PharMingen, Oxford, UK
CD80 (B7-1)	16-10A1	Armenian Hamster IgG2, κ	RPE	1 in 100	553769	BD Biosciences PharMingen, Oxford, UK
CD86 (B7-2)	GL1	Rat IgG2α, κ	RPE	1 in 100	553692	BD Biosciences PharMingen, Oxford, UK
CD8α (Ly-2)	53-6.7	Rat IgG2α, κ	FITC	1 in 100	553030	BD Biosciences PharMingen, Oxford, UK
CELLection Biotin Binder Kit			Biotin		115.33	Dynal Biotech ASA, Oslo, Norway
F4/80 Antigen	A3-1(F4/80)	Rat IgG2b	RPE	1 in 10	MCA497PE	Serotec, Oxford, UK
Fc block	2.4G2	Rat IgG2b, κ	-	1 in 100	553141	BD Biosciences PharMingen, Oxford, UK
H-2K^{b}	AF6-88.5	Mouse (Balb) IgG2a, κ	RPE	1 in 100	553570	BD Biosciences PharMingen, Oxford, UK

Antibody to	Clone	Isotype	Conjugated to	Working dilution	Catalogue no.	Supplier
I-A/I-E	M5/114.15.2	Rat IgG2b, κ	RPE	1 in 100	557000	BD Biosciences PharMingen, Oxford, UK
PDCA-1	JF05.1C2.4.1	Rat IgG2b	FITC	1 in 10	130-091-961	Miltenyi Biotec, Bergisch Gladbach, Germany
PDCA-1	JF05.1C2.4.1	Rat IgG2b	PE	1 in 10	130-091-962	Miltenyi Biotec, Bergisch Gladbach, Germany

2.1.5 Isotype-matched control antibodies for FACS analysis

Isotype	Clone	Conjugated to	Working dilution	Catalogue no.	Supplier
Hamster IgG1	G235-2356	APC	1 in 100	553956	BD Biosciences PharMingen, Oxford, UK
Hamster IgG1	A19-3	RPE	1 in 100	553972	BD Biosciences PharMingen, Oxford, UK

Isotype	Clone	Conjugated to	Working dilution	Catalogue no.	Supplier
Hamster IgG2, κ	B81-3	RPE	1 in 100	550085	BD Biosciences PharMingen, Oxford, UK
Mouse IgG2a, κ	G155-178	RPE	1 in 100	553457	BD Biosciences PharMingen, Oxford, UK
Rat IgG 2a, κ	R35-95	FITC	1 in 100	553930	BD Biosciences PharMingen, Oxford, UK
Rat IgG 2b, κ	A95-1	FITC	1 in 100	556923	BD Biosciences PharMingen, Oxford, UK
Rat IgG2a, κ	R35-95	RPE	1 in 100	554688	BD Biosciences PharMingen, Oxford, UK
Rat IgG2b, κ	A95-1	RPE	1 in 100	553989	BD Biosciences PharMingen, Oxford, UK

2.1.6 Antibodies used in confocal/immunofluorescence Studies

Antibody to	Clone	Isotype	Conjugated to	Working dilution	Catalogue no.	Supplier
Murine-IFN-α	F18	Rat IgG1	unconjugated	1 in 2	HM1001	HyCult Biotechnology b.v., Uden, The Netherlands
Murine-PDCA-1	JF05-1C2.4.1	Rat IgG2b	biotin	1 in 5	130-091-964	Miltenyi Biotec, Bergisch Gladbach, Germany
LCMV NP	NP-113	Mouse IgG2a	Cy5	1 in 10	-	Hybridoma cells were a gift from Dr Michael Buchmeier, The Scripps Research Institute, La Jolla, CA, USA
LCMV Arm E350	Antiserum immune ascitic fluid	Mouse antibodies	unconjugated	1 in 50	VR1271-AF	ATCC, LGC Promochem, Middlesex, UK

2.1.7 Secondary Conjugates and Dyes

Reagent	Isotype	Conjugated to	Working dilution	Catalogue no.	Supplier
Anti-rabbit IgG	Affinity isolated goat polyclonal	biotin	1 in 1000	B 8895	Sigma-Aldrich, Poole, UK
Streptavidin- Alexa Fluor 488	-	Alexa Fluor 488	1 in 2000	554060	BD Biosciences PharMingen, Oxford, UK
Anti-mouse polyvalent Ig (IgG1, IgA, IgM)	Affinity isolated goat polyclonal	FITC	1 in 200	F1010	Sigma-Aldrich, Poole, UK
Bo-Pro	-	-	1 in 200	B3587	Molecular Probes, Invitrogen, Paisley, UK
Anti-rat IgG (H+L)	Affinity purified donkey antibodies	Alexa Fluor 488	1 in 100	A21208	Molecular Probes, Invitrogen, Paisley, UK
Anti-mouse-IgG(H+L)-Alkaline	Affinity purified goat antibodies	Alkaline phosphotase	1 in 1000	1031-04	Southern Biotech, Cambridge Biosciences, Cambridge, UK

Reagent	Isotype	Conjugated to	Working dilution	Catalogue no.	Supplier
Phosphotase (AP)					
Streptavidin-AP	-	AP	1 in 1000	7100-04	Southern Biotech, Cambridge Biosciences, Cambridge, UK
TSA kit (HRP-streptavidin and AlexaFluor594 tyramide)	-	HRP-Streptavidin & AlexaFluor594 tyramide	1 in 100	T-20935	Molecular Probes, Invitrogen, Paisley, UK
ImmunoPure (A Plus) IgG Purification kit	Protein A			44679	Pierce, Rockford, IL, USA
AP conjugate substrate kit	-	AP		170-6432	BioRad Laboratories Ltd, Hemel Hempstead, UK

2.1.8 Isotypes matched antibodies for confocal/immunofluorescence, control sera and blocking reagents

Isotype	Clone	Conjugated to	Working dilution	Catalogue no.	Supplier
Rat IgG2b	A95-1	biotin	1 in 5	553987	BD Biosciences PharMingen, Oxford, UK
Rat IgG1	R3-34	unconjugated	1 in 2	559072	BD Biosciences PharMingen, Oxford, UK
Mouse IgG2a	G155-178	Cy5	1 in 10	555575	BD Biosciences PharMingen, Oxford, UK
Normal rabbit serum	-	unconjugated	1 in 100	-	Kind gift from Julie Lewis, Edward Jenner Institute, Compton, UK
Normal mouse serum	-	unconjugated	1 in 100	X0910	DakoCytomation, Glostrup, Denmark
Mouse on Mouse (M.O.M) Immunodetection kit	-	-	90µl in 15 ml	PK-2200	Vector Laboratories, Peterborough, UK

2.1.9 Antibodies used in type 1 IFN quantitation assays

Antibody to	Clone	Isotype	Conjugated to	Working dilution	Catalogue no.	Supplier
Mouse IFN-α	Polyclonal antibody	Rabbit polyclonal	unconjugated	1 in 6000	32100-1	PBL Biomedical Laboratories, Piscataway, NJ, USA
Mouse IFN-β	Polyclonal antibody	Rabbit polyclonal	unconjugated	7 Neutralization units per 1U IFN	AB2215	Chemicon International, Temecula, CA, USA
Mouse IFN-γ	F-3	Rat IgG2a	unconjugated	0.4μg/ml	HM1002a	HyCult Biotechnology b.v., Uden, The Netherlands

2.1.10 Plasmids

Plasmid	Protein	Vector	Selection marker	Provided by
p(9-29 ISRE) 4tkΔ(-39)lucter	Luciferase	pBR322	Ampicillin	Dr. Steve Goodbourn (St George's Hospital Medical School, University of London, London, UK)
pMX-CAT	CAT	pRSV-CAT	Ampicillin	Dr. Bryan Charleston (IAH, Compton, UK)
pCDNA-GFP	Green fluorescent protein (GFP)	pCDNA	Ampicillin	Dr. Mike Flint (University of Reading, UK)
pCDNA-6 V5-HisB	Blasticidin		Ampicillin	Invitrogen, Paisley, UK
pCAGGS-GP	LCMV-GP	pCAGGS	Ampicillin	All constructs provided by Dr. Juan Carlos de la Torre (The Scripps Research Institute, La Jolla, CA, USA)
pCAGGS-NP	LCMV-NP	pCAGGS	Ampicillin	
pCAGGS-ZII	LCMV-Z	pCAGGS	Ampicillin	

2.1.11 Sequences for Taqman primers and probes

Gene	Reference	Primers and Probe Sequence	Primer dilution *	Baseline and Threshold values
IFN-β	(Karaghiosoff et al. 2003)	Forward : ATGAGTGGTGGTTGCAGGC Reverse : TGACCTTTCAAATGCAGTAGATTCA Probe : FAM-AAGCATCAGAGGCGGACTCTGGGA - TAMRA	1:5	(3-15), 0.05
IFN-α	(Karaghiosoff et al. 2003)	Forward: CTGTGTGATGCAGGAACC Reverse: TCACCTCCCAGGCACTGA Probe : FAM-AGACTCCCTGCTGGCTGTGAGGACA - MGB-NFQ	1:2	(3-24), 0.08
IFN-α(non-α4)		Forward (for α 6,8,7,11): CACTGTGTTCCTGAGAGAGAAGAAAC Forward (for 5,2,1,13, pseudogene): CACTGTGTACCTGAGAGAGAAGAAAC Reverse: GGAAGACAGGGCTCTCCAGACT Probe : FAM-CACCTCCCAGGCACAGGGGCT-MGB-NFQ	1:5	(3-18), 0.03
Hypoxanthine ribosyl transferase HPRT	(Karaghiosoff et al. 2003)	Forward: TTGCTCGAGATGTCATGAAGGA Reverse: TGAGAGATCATCTCCACCAATAACTT Probe : FAM -CTGGAGGGCGTGCAGCGTGA - TAMRA	1:10	(3-15), 0.03
Mx		Forward: GGAATTACCAGGGTGGCTGTAG Reverse: TGGATGTATGTCTTGATAAGTCTCTTGA Probe : FAM- ACCAGCCTGCAGACATAGGACGCC-TAMRA	1:10	(3-15), 0.03

Gene	Reference	Primers and Probe Sequence	Primer dilution *	Baseline and Threshold values
LCMV-NP		Forward: CAGCATCTTCCTGCACCTTGT Reverse: ATCGGTGTGGCTTTGGACAT Probe : FAM- AATACCAACACACAGGCACATAAAAGGTGGC-TAMRA	1:10	(3-11),0.05
Influenza A	(van Elden et al. 2001)	Forward 1: GGACTGCAGCGTAGACGCTT Forward 2: CATTCTGTTGTATATGAGGCCCAT Reverse : CATCCTGTTGTATATGAGGCCCAT Probe : FAM- CTCAGTTATTCTGCTGGTGCACTTGCCA-TAMRA	1:2	(3-18),0.01
Sendai Z-NP	(Wagner et al. 2003)	Forward: CAGAGGAGCACAGTCTCAGTGTTC Reverse: TCTCTGAGAGTGCTGCTTATCTGTGT Probe : FAM- TGCATCATCAGTCACACTTGGGCCTAGTA-TAMRA	1:7.5	(3-8), 0.1

*dilution is from stocks of forward and reverse primers at 100pmol/μl each.

2.1.12 Viruses

Virus	Originally obtained from	Working stock grown in	Prepared by	Titre of working stock
LCMV Arm	Dr. Michael Oldstone (The Scripps Research Institute, La Jolla, CA, USA)	BHK cells	Mai Lee Wong	6×10^7 pfu/ml
LCMV Cl13	Dr. Michael Oldstone (The Scripps Research Institute, La Jolla, CA, USA)	BHK cells	Mai Lee Wong	5.6×10^7 pfu/ml
LCMV Docile	Dr. Rolf Zinkernagel (Institute for Experimental Immunology, University of Zurich, Zurich, Switzerland)	BHK cells	Lian Ni Lee	1.2×10^7 pfu/ml
Influenza A/PR/8/34	Dr. Sam Hou (Edward Jenner Institute, Compton, UK)	Allentoic fluid	Charles River SPAFAS, North Franklin, USA	512 HA units/0.05ml
EMCV	ATCC virus collection,	BHK cells	Dr. Mike Riffkin	5×10^8 pfu/ml

Virus	Originally obtained from	Working stock grown in	Prepared by	Titre of working stock
	LGC Promochem, Middlesex, UK			
Sendai Z	Dr. John Macauley (IAH, Compton, UK)	10-day old embryonated chicken eggs	Dr. John Macauley	~10^7 p.f.u/ml
HSV-1	Dr. Terry Hill (University of Bristol, Bristol, UK)	DM-21 cells	Miranda Ashton	1x10^7 p.f.u/ml

2.1.13 Mammalian cell lines

Cell line	Catalogue No.	Description	Supplier
BalbC17	-	Mouse fibroblast cell line derived from Balb/c mice. This cell line was transfected for IFN reporter bioassays and infected with LCMV for *in vitro* chronic infection studies	Laboratory stocks, originally obtained from ATCC, LGC Promochem, Middlesex, UK
Vero	ATCC: CCL-81	An adherent cell line derived from African green monkey kidney cells and used in viral plaque assays	Laboratory stocks, originally obtained from ATCC, LGC Promochem, Middlesex, UK

Cell line	Catalogue No.	Description	Supplier
BHK	ATCC: C-13	An adherent cell line derived from baby hamster kidney cells and used to grow up stocks of LCMV Docile virus.	Laboratory stocks, originally obtained from ATCC, LGC Promochem, Middlesex, UK
L929	ATCC: CCL-1	Mouse fibroblast cell line used in IFN bioassays	Laboratory stocks, originally obtained from ATCC LGC Promochem, Middlesex, UK
FSDC	-	Semi-adherent mouse DC line used in chronic LCMV experiments	Dr. Peter Beverley, Edward Jenner Institute, Compton, UK
D2SC/1	-	Semi-adherent mouse DC line used in chronic LCMV experiments.	Dr. Peter Beverley, Edward Jenner Institute, Compton, UK

2.1.14 Mice

Female C57BL/6 mice 8-12 weeks of age supplied by Charles River (Margate, UK) were used for all *in vivo* experiments. BM-DC were generated from C57BL/6 mice obtained from the SPF animal unit (IAH, Compton, UK)

2.2 Propagation of cell lines

BHK cells were grown in BHK medium supplemented with 10% foetal calf serum (FCS), 10% tryptose-phosphate broth and 1 μg/ml (P/S) in a 37°C, 5% CO_2 incubator. They were subcultured when confluent by washing the monolayer with sterile 0.9% saline and adding 0.05% trypsin/0.02% EDTA solution.

Vero cells were grown in RPMI 1640 with 7% FCS and 1 μg/ml P/S and maintained in a 37°C, 5% CO_2 incubator. They were subcultured as above.

Untransfected BalbCl7 cells were grown in Eagle's MEM supplemented with 7% low endotoxin FCS and 1 μg/ml P/S at 37°C in 5% CO_2. Stably transfected cells were cultured in growth media supplemented with 8 μg/ml blasticidin. Cells were subcultured as described above.

L929 cells were grown in DMEM supplemented with 7% FCS and 1 μg/ml P/S at 37°C, 5% CO_2. Cells were subcultured as described above.

FSDC cells were grown in RPMI 1640 medium supplemented with 10% GFCS and 1 μg/ml P/S at 37°C in 5% CO_2. Confluent cells were resuspended into spent media and cultered into new flasks with fresh media.

D2SC/1 cells were grown in RPMI 1640 medium supplemented with 10% GFCS and 1 μg/ml P/S at 37°C, 5% CO_2. Confluent cells were subcultured as described for FSDC cells.

2.3 Preparation and assay of viruses

2.3.1 Preparation of working stocks of LCMV Docile

From an aliquot of LCMV Docile (P1) provided by Dr Rolf Zinkernagel (Institute of Experimental Immunology, University of Zurich, Switzerland), master stocks (P2) and working stocks (P3) were generated. BHK cells were set up in T150 flasks (TRP, Trasadingen, Switzerland) flasks at ~$5x10^6$ cells per flask in a volume of ~30 ml of complete growth medium and grown at 37°C in a 5% CO_2 environment overnight. Cells were infected with a master stock of virus at a multiplicity of infection (m.o.i.) of 0.1 pfu/cell in 4 ml serum-free media per flask. After a one hour incubation period at 37°C/ 5% CO_2, 20 ml of supplemented complete growth medium was added to each flask and the cells were then incubated for a further two days. After this time the virus containing supernatant was harvested, and any cell debris removed by centrifugation at 1000g for 20 mins at 4°C Sorvall RT7 Plus (Sorvall, Newtown, USA). Aliquots of the resulting virus working stock were then made and stored at -80°C. The virus titre of the stock was determined by immunoplaque assay as described below.

2.3.2 Plaque assay for titration of LCMV Arm

LCMV Arm and Cl13 were titrated by plaque assay on Vero cells as described by Dutko and Oldstone (Dutko and Oldstone 1983). Briefly, Vero cells were seeded into 6-well plates at $3x10^5$ cells per well in 3 ml of growth medium and incubated overnight. Virus-containing test samples were serially diluted from 10^{-2} to 10^{-8} in serum free RPMI 1640. Supernatant was removed then replaced with 500 µl of each virus dilution and incubated for 1 hour. Cell monolayers were subsequently overlaid with 3 ml agar/199 media per well. Plates were wrapped in foil and incubated in a

37°C, 5% CO_2 incubator for 6 days. Monolayers were then fixed with 8% formaldehyde in PBS for 1 hour and subsequently stained with 0.1% crystal violet to visualise the plaques.

2.3.3 Immunoplaque assay for the titration of LCMV Docile

LCMV Docile was titrated on Vero cells as described above except that the monolayers were infected for 4 days and the plaques were visualised by immunostaining. Plates were fixed with formalin as described above and then fixed with 100% methanol for 20 minutes at -20°C. The monolayers were blocked with PBS-3%BSA for 1 hour at room temperature, washed with 0.9% saline and probed with anti-LCMV antibody (either anti-LCMV ascites VR1271-AF at a 1/50 or 1/100 dilution or biotin anti-NP monoclonal antibody at 1/12 dilution in PBS-1%BSA, at 500 µl per well. After 1 hour incubation at room temperature plates were washed and bound antibodies detected with either 500 µl anti-mouse-AP conjugate or streptavidin-AP (at 1/1000 dilution). Conjugate was incubated for 1 hour at room temperature before plaques were detected and visualised with AP substrate. Foci of infection appeared to be large, circular and with a defined edge.

2.3.4 Infection of mice with LCMV

Female C57BL/6 mice at 8-12 weeks of age were infected i.v with LCMV Arm at $5x10^5$ plaque forming units (p.f.u.) diluted in 200 µl serum-free RPMI media or LCMV Cl13 or LCMV Docile at $5x10^6$ p.f.u. in 200 µl serum-free RPMI media. They were housed in Category 3 containment facilities and provided with food and water ad labitum. All animal studies were carried out in accordance with UK Home Office regulations and were approved by the site ethical review committee.

2.4 Plasmid preparation

2.4.1 Plasmids for IFN-reporter gene assays

1. p(9-29 ISRE) 4tkΔ(-39)lucter (also known as pISRE-luc)

The plasmid contains 4 IFN-stimulated response elements (ISRE) linked to the luciferase reporter gene (King and Goodbourn 1998) and can be used for the detection of biologically active IFN-α and -β.

2. pMX-CAT

This plasmid contains the CAT gene under the control of an IFN-inducible *Mx*-promoter. pMX-CAT was derived from pRSV-CAT by inserting the human Mx-promoter region upstream of the CAT gene (Ronni et al. 1998). The *MX*A promoter is induced by IFN-α/β, with high, sustained expression even after transient exposure to type 1 IFNs (Baigent et al. 2002) .

3. pcDNA6 V5-HisB (Invitrogen)

The plasmid encodes a blasticidin resistance gene and was co-transfected with the IFN responsive constructs to provide a selectable marker for stably transfected cells.

4. pCDNA-GFP

This is a control plasmid that encodes for green fluorescent protein (GFP). It was used as a positive control to determine the efficiencies of transfection.

All of these plasmids, apart from plasmid pcDNA-GFP, were transformed into *rec*A1$^-$ competent cells, purified and checked by Julie Lewis.

Plasmid pCDNA-GFP was transformed into DH5α competent cells, purified and checked by Rachel Owen.

2.4.2 Plasmids containing individual LCMV genes

pCAGS-GP, NP and Z encode the full length sequence of the GP, NP and Z proteins of LCMV strain Arm 53. The proteins were inserted downstream of a chicken γ globulin promoter.

2.4.3 Transformation of LCMV plasmids

pCAGS-GP, NP and Z were transformed into DH5α competent cells using a standard heat-shock method. 50 µl of competent cells were aliquoted into chilled eppendorf tubes. 2 µl of plasmid DNA (18-32 ng DNA) were added to the cells and incubated on ice for 30 mins. The cells were given a heat-shock by immersion in a 37°C water-bath for 20 seconds, then were placed on ice for 2 mins. 950 µl of Luria Bertani (LB) medium was added and incubated for 1 hour at 37°C. 100 µl of the cell mixture was plated out on LB agar plates containing 100 µg/ml ampicillin and incubated overnight at 37°C in an InnOva 4230 shaker incubator to select for transfected colonies. 5 single colonies from each plasmid were selected at random and inoculated into 5 ml LB broth cultures containing 100 µg/ml ampicillin and incubated overnight at 37°C in an InnOva 4230 incubator with shaking at 225 rpm.

2.4.4 Plasmid purification

Plasmids were purified using a Qiagen Plasmid Miniprep kit according to the manufacturer's instructions. Plasmid DNA was eluted in 50 µl TE buffer (0.5M EDTA, 1 ml Tris(hydroxymethyl) aminomethane) in H_2O at pH 7.4) and the identity of each plasmid was checked by restriction enzyme digest followed by gel

electrophoresis of the digested products to confirm the presence of the desired plasmids.

The following restriction enzymes were used to identify purified plasmids:-

Plasmid	Restriction enzyme(s)	Expected products
pCAGS-GP	HindIII	Single band 6.2kb
pCAGS-NP	EcoRI and BglII	1.6kb and 4.8kb bands
pCAGS-Z	EcoRI	Single band 4.9-5kb

1 µl of plasmid DNA was digested for 1 hour at 37°C with the respective restriction enzymes and the products resolved by electrophoresis on 1% TAE agarose gels containing ethidium bromide (1 µl per 10 ml gel) together with 1 kb Plus and 100 bp DNA ladders in Biorad subcell electrophoresis chambers (Biorad Laboratories, Hemel Hampstead, UK) and visualised under UV light (Jencons Forest Row, UK).

The colony displaying the highest expression of the relevent plasmid (most intense band on the gel) was selected for generation of glycerol stocks. The selected colonies were inoculated into 3ml of LB broth cultures containing 100 µg/ml ampicillin and incubated for 8 hours at 37°C in an InnOva 4230 incubator (Fischer Scientific, Loughborough, UK) with shaking at 225 rpm. Cultures were removed from incubation and mixed with 3 ml glycerol. This 1:1 mixture of culture:glycerol was aliquoted into cryotubes at 2 ml/tube and stored at -80°C.

2.4.5 Large-scale Preparation of Plasmid DNA

Large-scale preps of the IFN-reporter plasmids p(9-29 ISRE) 4tkΔ(-39)lucter and pMX-CAT, the selection plasmid pcDNA6 V5-HisB, the control plasmid pCDNA-GFP and the LCMV plasmids pCAGS-GP, NP and Z were prepared. Frozen glycerol

stocks were streaked onto LB agar plates containing 100 μg/ml of ampicillin from streaking relevant frozen glycerol stocks. A single colony for each plasmid was selected and inoculated into 3 ml of LB broth culture containing 100 μg/ml ampicillin and this was incubated for 8 hours at 37°C in an InnOva 4230 incubator with shaking at 225 rpm. 1ml of culture was then used to inoculate 100 ml of LB broth culture containing 100 μg/ml ampicillin and incubated overnight at 37°C in an InnOva 4230 incubator with shaking at 225 rpm.

Bacterial cells were pelleted from the broth by centrifugation for 15 min at 6000g at 4°C using a Beckman J-301 (Beckman Coulter, Fullerton, CA, USA) centrifuge. Plasmid was purified from the cell pellets using the Endofree maxiprep purification kit, following the manufacturer's instructions. Precipitated plasmid DNA was resuspended in 500-1000 μl of TE buffer. The purity and quantity of each plasmid was determined by UV spectroscopy on a GeneQuant pro RNA/DNA calculator (Amersham Pharmacia Biotech, Cambridge, UK) by measurement of the absorbance at 260 nm and 280 nm. The 260 nm/280 nm ratio for all plasmids was always ~1.8, indicating a pure DNA prep.

2.5 Measurement of type 1 IFN Protein or Activity

2.5.1 IFN-alpha ELISA

Two methods were employed to determine concentrations of IFN-α protein. The first method used a commercially available ELISA specific for murine IFN-α (PBL laboratories), according to the manufacturer's protocol. Briefly, samples were added in duplicate to coated 96-well ELISA plates in a volume of 100μl/well, in parallel with known concentrations of IFN-α solution provided with the kit, to construct a standard curve. When appropriate, the samples and standards were diluted in dilution

buffer prior to addition. The plate(s) were sealed and incubated for 1 hour, then washed with 1x wash buffer. 100 µl of IFN-α antibody solution at working concentration was then added to each well and the plate(s) were incubated for 24 hours at 24°C. Unbound antibody was then removed and the wells were washed again with 1x wash buffer prior to addition of 100µl/well of 1x HRP conjugate and incubated for 1 hour at 24°C. The plates were washed and then 100µl/well TMB solution was added and allowed to develop for 15 mins in the dark. The reaction was stopped with 100µl stop solution and the absorbance was determined by UV spectroscopy at 450nm on a Spectramax 340 plate reader (Molecular Devices, Winnersh, UK). A standard curve was constructed using readings from the known IFN-α concentrations and sample calculations were performed using the Softmax Pro analysis package (Molecular Devices).

Alternatively, IFN-α was quantitated using a laboratory-based protocol. 96-well plates were coated with anti-IFN-α monoclonal (Clone F18, Hycult) diluted 1/100 in coating buffer, 0.1M $NHCO_3$ pH8.4, at 100µl /well. After an overnight incubation at 4°C the plates were blocked with 200µl /well PBS-2 %FCS. After a 2 hour incubation at room temperature, blocking buffer was removed, then 50µl/well samples or recombinant IFN-α at known concentrations, which were either serum or cell supernatants diluted in blocking buffer, were added in duplicate and incubated on a shaker for 4 hours at room temperature. Samples were aspirated and the plate was washed once with washing buffer (PBS-0.5% Tween 20), before addition of 100µl /well polyclonal anti-IFN-α detection antibody diluted 1/9000 in blocking buffer. After an overnight incubation at 4°C, the plate was rinsed 4 times with washing buffer and biotinylated goat-anti-mouse Ig diluted 1/10000 in blocking buffer was added at 100µl /well for one hour at room temperature. The plate was washed 4 times with

washing buffer before 100µl /well streptavidin-HRP was added for 2 hours at room temperature. The plate was washed 6 times with washing buffer prior to addition of 100µl /well OPD substrate solution, then allowed to develop for about 10 mins before the absorbance was determined by UV spectrometry at 450nm on a Spectramax 340 plate reader (Molecular Devices, Winnersh, UK). Standard curve was constructed using readings from recombinant IFN-α and sample calculations were performed using the Softmax Pro analysis package (Molecular Devices).

2.5.2 IFN Bioassay for antiviral activity

Type 1 IFN antiviral activity was assayed using a bioassay based on the method original described by Familletti *et a*l (Familletti et al. 1981). 100µl of sample was added to the first wells of each row of a 96-well plate containing 50µl of complete media in the other wells. Serial 2-fold dilutions were performed across the plate by transferring 50µl from well 1 into the next well along the row until the end. A standard curve of recombinant IFN-α was set up on the top row of every plate by adding 100U of recombinant IFN-α into well 3 of the top row and diluting 2-fold across the remainder of the row as for the samples. The first two wells were left as positive (no virus) and negative controls (no IFN). If neutralizing anti-IFN- α ,β or γ antibodies were added the samples would be incubated with the antibody for 30 mins at 37°C. A single-cell suspension of L929 cells at a concentration of 2 $x10^6$ cells/ml was made up using supplemented growth media. 50µl of the suspension was added to each well, making the final cell concentration $1x10^5$ cells/well, and then the plates were incubated at 37°C in 5% CO_2 overnight. The following day, the medium was removed and the cells were rinsed with PBS then infected with 10^4 p.f.u encephalomyocarditis virus (EMCV) in DMEM-0.5% BSA at 100µl /well. After a 16

hour incubation in 37°C, 5% CO_2, the virus was removed and the wells rinsed with PBS, fixed with 8% formalin for 1 hour and stained with 0.1% crystal violet. The first well in each sample dilution series that exhibited the 50% cytopathic effect observed in the IFN alpha standard was defined as the endpoint and the IFN titre was calculated from this by comparison to the standard curve (titres were expressed as U/ml).

2.5.3 Generation of cell lines stably transfected with IFN-responsive reporter plasmids and their use in assaying IFN-α/β activity

2.5.3.1 Transfection of reporter gene constructs

Murine BalbC17 cell lines were stably transfected with the plasmids pISRE-luc, pMX-CAT. They were plated into 6-well plates and when at 70-80% confluency were transfected with GeneJuice Transfection Reagent following the manufacturer's protocol. Briefly, medium was removed and replaced with medium containing plasmid DNA mixed with GeneJuice reagent. After an overnight incubation at 37°C in 5% CO_2, the medium was removed and replaced with fresh growth medium and incubated for a further 24-48 hours before reporter gene expression was assesed. Reporter plasmids were co-transfected with pcDNA6 V5-HisB at a ratio of 1:1 respectively. An aliquot of cells were transfected with pCDNA-GFP in parallel using the same conditions in order to assess transfection efficiency.

2.5.3.2 Selection of stably transfected cells

Approximately one week after transfection growth medium was supplemented with 10μg/ml of Blasticidin S. Resistant colonies were observed and these were maintained within the selection media. After further expansion, the resistant colonies were trypsinized and pooled together. A bank of cells was frozen down while the rest were

gradually expanded into 60mm dishes, then T25 and finally T75 culture flasks. Stably transfected cells were later cloned by limiting dilution, seeding cells into 96-well plates at a concentration of 1.2 cells/well. Single cell colonies were identified. Clones were expanded into 24-well plates and tested to identify those giving the best response to IFN-α.

2.5.3.3 IFN assay using reporter plasmids

Transfected cells were seeded into either 6-well plates at $1x10^6$ cells/well or $2.5x10^5$ cells/well, 24-well plates at $6x10^4$ cells/well or 48-well plates at $0.5\text{-}2x10^4$ cells per well and the plates were incubated overnight at 37°C in 5% CO_2. The medium was removed and samples were added in a volume of 1ml/well for 6-well plates, 500µl/well for 24 well plates or 250µl/well for 48-well plates. The plates were then incubated for 18-24 hours. All assays included a medium only control and IFN-α and/or -β standard curves. In other assays polyclonal antibodies against murine IFN-α or IFN-β was included in the assay medium at 100 Neutralizing Units (N.U). All variables were assayed in duplicate. Reporter gene expression was quantified as described below.

2.5.3.4 Detection of luciferase reporter gene expression

Expression of luciferase was quantitated using a commercially available Luciferase Assay System which measures bioluminescence emission catalyzed by luciferase. The medium was aspirated from the assay wells and the cells were lysed with 70µl of lysis buffer. 35-50µl of lysate was transferred into opaque 94-well microtitre plates and 50µl luciferase substrate added. Luminescence was quantified on a Wallac Victor^2

1420 multilabel counter (PerkinElmer, Beaconsfield, UK). Results from these assays were expressed as counts per minute (cpm).

2.5.3.5 Detection of CAT reporter gene expression

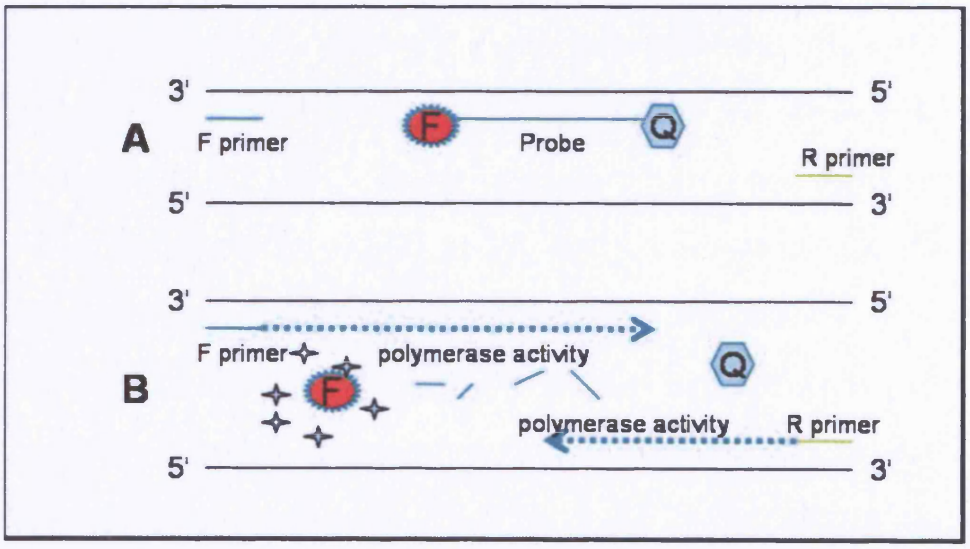
CAT expression was determined using a commercial ELISA kit. Briefly, cells in assay wells were washed with cold PBS and lysed with 75µl lysis buffer for 20 mins. All the lysate was transferred to individual wells of the CAT-ELISA plate and assayed according to the manufacturer's instructions. Briefly, 96-well plate-sized strips precoated with anti-CAT antibody was rehydrated and blocked. Blocking buffer was removed and samples were added, diluted in sample buffer. After 4 hour incubation at room temperature, the samples were removed, the wells were washed and anti-CAT-antibody conjugated to dixogenin (DIG) was added. The readings were amplified by detecting bound anti-CAT antibody with anti-DIG antibody conjugated to peroxidase (POD). POD substrate was added for colourimetric assay. The absorbance of the samples was measured at 405nm read using a Spectra Max 340 reader (Molecular Devices). Results were analysed with Softmax Pro software (Molecular Devices) and expressed as absorbance at 405nm.

2.6 Quantitation of Type 1 IFN mRNA

2.6.1 Introduction

The reverse-transcriptase polymerase chain reaction (RT-PCR) is a method whereby existing mRNA templates of a given gene are amplified through *in vitro* gene transcription. The mRNA is first converted into DNA by RNA-dependent reverse transcription. On the templates of interest, DNA between two specific oligonucleotide primers is amplified by thermocycling in the presence of Taq DNA polymerase and

free nucleotides. As the amount of amplified product at the end of the experiment correlates with the initial number of templates in the input sample, monitoring product formation during the linear phase of amplication enables quantitative analysis of the levels of template in a sample. A schematic of the Taqman real-time PCR detection principle is shown below. In a Taqman PCR reaction, a Taqman probe, consisting of an oligonucleotide labelled with a reporter fluorochrome on one end and a quencher dye on the other is designed to specifically recognize an internal sequence within the amplified DNA region. In its intact form (A), the net output of fluorescence signal is zero as the fluorescence emission on one end will be quenched by the quencher dye. This probe will bind to the amplified sequence during the annealing phase. During the extension phase of the amplification (B), the 3'-5' nuclease activity of the Taq polymerase will degrade the probe as it extends the new DNA chain. The separation of the reporter and quencher results in an increase in reporter emission. The amount of fluorescence released is directly proportional to the amount of product generated in each PCR cycle and can be applied as a quantitative measure of PCR product formation.



The principle of quantitative Taqman PCR.

2.6.2 Design of Taqman Primers and probe

Primers and probes that were specific for the detection of murine IFN-β and IFN-α4 and the housekeeping gene HPRT have been published in the literature, optimized and used to quantitate IFN-β and IFN-α4 mRNA transcript levels (Karaghiosoff et al. 2003). Oligonucleotides and Taqman FAM-TAMRA probes for IFN-β, IFN-α4 and HPRT were synthesised according to the published sequences, and used in accordance to the published experimental condition. The primers and probes for detection of murine Mx were designed and optimized by Elizabeth Balman of the Memory Group, Edward Jenner Institute, Compton. In order to design Taqman primers and probe that could detect all IFN-α subtypes except for IFN-α4, genomic or cDNA sequences of murine IFN-α2, 4, 5, 6, 8,9, 11, 12, 13 subtypes and IFN-β were downloaded from the GenBank database. The accession number for each sequence is listed in the following table:

Table 2.1 Type 1 IFN genes aligned

Gene	Genbank Accession
IFN-α family	gi|194094|gb|M68944.1|MUSIFN[194094]
IFN-α 2 mRNA	gi|6754291|ref|NM_010503.1|[6754291
IFN-α4 mRNA	gi|6754293|ref|NM_010504.1|[6754293]
IFN-α5 gene	gi|51546|emb|X01971.1|MMIFNA5[51546]
IFN-α6 gene	gi|927431|emb|X01972.1|MMIFNA6[927431]
IFN-α8 gene, full coding sequence (cds)	gi|220441|dbj|D00460.1|MUSIFNA8[220441]
IFN α (1-9) gene, complete cds	gi|194100|gb|M13660.1|MUSIFNA1A[194100]
IFN-α11 precursor gene, complete cds	gi|29469012|gb|AY225954.1|[29469012
IFN-α12 mRNA, complete cds	gi|28628329|gb|AY190046.1|[28628329]
IFN-α 13 mRNA, complete cds	gi|28628331|gb|AY190047.1|[28628331]
IFN-β mRNA	gi|6754303|ref|NM_010510.1|[6754303]

Multiple sequence alignment was carried out using the ClustalW program (Pearson and Lipman 1988) (website: http://www.ebi.ac.uk/clustalw). Conserved regions of IFN-α2, 5, 6, 8, 9, 11, 12 and 13 were identified and downloaded onto the Primer Express software program (PE Applied Biosystems, Warrington, UK) and primers and probes were designed that recognized the conserved regions and conformed to the following constraints:

Primers – must not have >2 cytoseine (C)/guanine (G) in the last 5 nucleotides (i.e. on the

3' end)

- forward and reverse primers should have equal annealing temperatures (Tms) where possible

Probe – must not start with a G on the 5' end

- must have more C residues than G residues

- Tm must be 7-10°C higher than the primers

Suitable primers and probes were selected and these were checked by BLAST (Altschul et al. 1990) to ensure they were specific for the IFN-α subtypes and did not correspond to sequences in the IFN-β gene, other murine genes or LCMV. Primers specific for IFN-α4 were checked in a similar manner, with the multiple sequence alignment of the IFN-α genes used to ensure the IFN-α4 primers recognised a unique region of the IFN-α4 gene and would not cross react with other IFN-α subtypes. The specificity of the IFN-β primers and probes were likewise confirmed.

Primer and probe sequences specific for LCMV envelope glycoprotein were also designed using the Primer Express Software program in collaboration with Miranda Ashton. The reference sequence for LCMV S (reference M20869, gi 1331358) was used primers and probes were synthesized that were specific for the GP-C viral

protein, and did not cross react with murine genes. The sequence of primers and probes that detected the Influenza A and Sendai virus were obtained from the literature (van Elden et al. 2001; Wagner et al. 2003). See Figure 3.13 in the next chapter for the full sequence of all the primers and probes used.

2.6.3 Purification of total RNA from tissue samples

Pieces of spleen were snap frozen in liquid nitrogen at the time of excision and stored at -80°C until purification. Tissues were homogenized using dounce (IKA, Fischer Scientific, Loughborough, UK) or bead mill (Retsch, Leeds, UK) homogenizers in the presence of 300µl of RLT buffer from the Qiagen Rneasy RNA Mini Isolation kit supplemented with 1% β-mercaptoethanol per 30mg tissue. Homogenized tissue was loaded onto a Qiashredder column and centrifuged to shear the genomic DNA. Total RNA was subsequently extracted from the lysate using the animal tissue protocol of the Rneasy Mini RNA Isolation kit. Briefly, the lysate was mixed with 70% molecular biology grade ethanol and added to the RNA isolation column. Genomic DNA contamination was removed by on-column DNase1 cleanup followed by washes with RW and RPE buffers. Total RNA was eluted from the column in 50µl of nuclease-free water and the purity and concentration were determined by UV spectroscopy (Pharmacia) using an aliquot of total RNA diluted in nuclease-free water and blanked with water. The 260nm/280nm ratio was always within the 1.8-2.0 range, indicating samples were not contaminated with protein.

2.6.4 Purification of total RNA from cultured cells

For semi-adherent cells grown in 6 or 24 well plates, spent media was removed and collected. The monolayer was washed with PBS and the wash buffer also collected.

The medium and wash buffer was centrifuged at 1500rpm for 5 mins to pellet the cells. This was resuspended in 300µl RLT+ 1% β-mercaptoethanol buffer and returned to the monolayer. If the semi-adherent cells were cultured in 96-well plates, the entire plate was centrifuged at 1500rpm for 5 mins to pellet the cell. Spent media was removed and 300 µl RLT+ 1% β-mercaptoethanol buffer was added to the cell pellet. If the cells were an adherent monolayer, the spent media was removed and 300µl RLT+ 1% β-mercaptoethanol buffer was added directly to the adherent monolayer. Lysates were collected and purified using the method used to extract total RNA from tissue samples or was stored at -80°C until purification.

2.6.5 Taqman PCR reaction

For initial optimization assays, Taqman qPCR reactions were set up containing 2.5µl of 10x Taqman universal Mastermix (proprietry formulation consisting of optimized concentrations of AmpliTaqGold DNA Polymerase, AmpErase Uracil-N-glycosylase, deoxynucleotides (dNTPs), passive reference dye and buffer components) 10-fold serial dilutions (top concentration 0.2µg/µl) of genomic DNA from wild-type C57Bl/6 mice as template, various concentrations of primers and labelled probes and enough nuclease-free water to make up a 25µl reaction volume in 96-well optical reaction plates (ABGene). Samples were run and analyzed on the ABI Prism 7700 Single Reporter Detection System 1.5 (Applied Biosystems, Warrington, UK) using the following cycling conditions:

AmpliTaqGold Activation	10 min 95°C	
PCR amplification	15 sec.95°C 1 min. 60°C	40 cycles

Results were analysed as described below.

2.6.6 Taqman qRT-PCR reaction

Expression levels of a selection of IFNβ, IFN-α4 subtype, all IFN-α subtypes and the IFN-induced gene, Mx mRNA transcripts were determined. When purified RNA could be quantitated by UV spectroscopy, 300-500ng of total RNA was used as template for duplicate Taqman real-time qRT-PCR reactions with primers and probes detailed in Table 2.11. In some cases, the amount of RNA purified was not sufficient for quantitation, e.g. total RNA from $CD11c^{+}$ $B220^{+/-}$ cells sorted from the spleen of infected mice and here, the entire sample was divided equally between the qRT-PCRs tested. The samples were amplified in 1x Reverse-Transcriptase qPCR™ Master Mix consisting of dNTPs, HotGoldstar DNA polymerase, 5mM $MgCl_2$, stabilizers, ROX passive reference dye, 0.25U/μl Euroscript reverse transcriptase and 0.1U/μl RNAse inhibitor plus respective primers and probes (see Table 2.x for concentrations) in a 25μl reaction volume. Reactions were set up in 96-well optical reaction plates. Samples were run and analyzed on the ABI Prism 7700 Single Reporter Detection System 1.5 (Applied Biosystems) using the following cycling conditions:

Initial reverse transcriptase step	30 mins 48°C	
Hot GoldStar activation	10 min 95°C	
PCR amplification	15 sec.95°C 1 min. 60°C	40 cycles

Taqman qRT-PCR assays were also conducted to determine levels of a selection of viruses, including LCMV, Influenza A/PR/8/34 and Sendai. The reactions were set up as described for the IFN-α/β Taqman qRT-PCR reactions using similar amplification conditions apart from the Influenza A qRT-PCR which was run for 45 cycles instead of the usual 40 cycles.

2.6.7 Data Analysis

2.6.7.1 Setting the Baselines and Threshold values

The ABI Prism sequence detector monitors amplification of PCR products by quantitatively analyzing fluorescence emissions. The reporter dye (FAM) signal is measured against the internal reference dye (ROX) signal to normalize for non-PCR related fluctuations in the fluorescence in each well to give the normalized reporter value R_n at every PCR cycle. The baseline and threshold values must be properly set in order to determine when the target amplification is sufficiently above the background signal. The level of fluorescence in a well at a given cycle during PCR generally corresponds to the amount of target RNA present in the sample at the start. However, during early cycles of PCR, prior to significant accumulation of the target amplicon, background fluorescence levels can fluctuate as a result of changes in the reaction medium. The background signal in all wells during a given number of PCR cycles is used to determine the baseline fluorescence across the plate. Usually, the baseline flurorescence is the fluorescence generated between PCR cycle 3 and one or two cycles before the earliest amplification emerges. When the baseline is set correctly, a plot of the linear view of ΔR_n (R_n-background fluorescence) vs PCR cycle number will show a flat line at 0.00 ΔR_n between the set baseline cycles followed by increasing ΔR_n values in subsequent cycles. If that is not the case, the stop value will be re-set until a flat line is generated. The Sequence Detector software sets the baseline as the value in PCR cycles 3 to 15. In practice, the default setting is not altered if the first reaction emerges after cycle 15 unless a good baseline is not generated. The plot is then switched to log view and the threshold value is set manually in the region of exponential amplification, i.e. the linear portion of the plot

that maximises the sensitivity and precision of the assay. The threshold cycle (Ct) value reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold.

2.6.7.2 Relative quantitation of mRNA expression

To quantitate and compare the relative transcription levels of target mRNAs in different samples, the levels were 'normalised' to the levels of housekeeping gene HPRT in each sample. The difference between the Ct value of the housekeeping gene HPRT and the Ct value of the target genes, i.e. IFN-β, IFN-α4, IFN-α(non-α4) and Mx, in the same sample was determined ($\Delta Ct = Ct_{hprt} - Ct_{target}$). As expression of the housekeeping gene is believed to be constant, higher ΔCt values thus reflect the presence of increased target mRNA in the samples.

2.7 *In vivo* responses to LCMV infection

2.7.1 Collection of spleen and blood from LCMV-infected C57Bl/6 mice

Mice were infected with LCMV as described in section 2.3.4. Mice were culled at timepoints between 6 hours and 6 weeks post infection and blood and spleens were collected.

Blood was collected by cardiac puncture and was allowed to clot at room temperature for about 1 hour before centrifugation at 6000g for 10 mins. Separated serum was removed and stored in aliquots at -80°C before use. Spleens were removed, cut into pieces and divided between cryotubes, snap-frozen in liquid nitrogen and stored at 80°C for subsequent RNA extraction, or immersed in OTC medium in tissue moulds, snap frozen in liquid nitrogen or dry ice and then stored at -20°C for subsequent cryosectioning.

2.7.2 Isolation and Analysis of murine splenic DC subpopulations

2.7.2.1 Isolation of murine splenic dendritic cells

Spleen DCs were obtained using a variation of the method described by Vremec *et al* (Vremec et al. 1992). 9-10 spleens were pooled from groups of mice at various timepoints post-LCMV infection and perfused with 200µl per spleen of DNase (325KU/ml):Collagenase (1mg/ml, type III) diluted in RPMI supplemented with 10% GFCS, 0.1mM EDTA pH7.2, 100U/ml polymyxin B and penicillin-streptomycin. After a 30 min incubation the spleens were mashed and passed through a 0.45µm sieve into a 50ml centrifuge tube containing RPMI-supplemented medium. Cells were pelleted by centrifugation and then resuspended in 3ml Nycoprep and overlaid onto 3ml Nycoprep in a 15ml centrifuge tube for density separation. The tubes were centrifuged for 20 mins at 2,000 x g in a Sorval RT7plus centrifuge. All the cells in

the upper zone were collected and transferred into a fresh 50ml centrifuge tube and washed twice with 20ml RPMI supplemented medium. After the second wash, the cell pellet was resuspended in RPMI-supplemented medium to a concentration of $1x10^8$ cells/ml, and CD11c MACS beads were added at a concentration of 100µl per 10^8 cells. This was incubated for 15 mins on ice, and then the cells were washed with 20mls supplemented RPMI to remove unbound beads. The cell pellet was resuspended in 5 ml supplemented RPMI and passed through an equilibrated CD11c MACS bead positive selection LS column placed on a magnet. The column was washed twice and then the cells were eluted with supplemented RPMI medium.

2.7.2.2 Analysis of expression of non-DC markers by $CD11c^+$ cells isolated from murine spleen

To determine the proportion of $CD11c^+$ cells isolated from the spleens of uninfected and infected mice that expressed markers characteristic of non-DC cell lineages, isolated $CD11c^+$ cells were co-stained with antibodies to CD11c, MHC Class II and either CD3 (a T-cell marker), CD19 (a B cell marker), CD14 (a monocyte marker), DX5 (expressed by many NK cells) or F4/80 (expressed by most mature macrophages). $CD11c^+$ cells were isolated from mouse spleens as described in the section above. After another wash, the positively selected cells were blocked with Fc block (1µl per 10^6 cells) on ice for 10mins, before cells were pelleted by centrifugation to remove unbound FC-block. Cells were resuspended in either supplemented RPMI media or FACS buffer (PBS, 2% FCS and 0.1%NaN_3) and plated into V-bottomed plates at $1x10^5$ cells per well and triple stained with anti-$CD11c^+$-APC, anti- MHC Class II-FITC and PE-conjugated anti-CD3, anti-CD19, anti-CD3, anti-DX5 or anti-F4/80 antibodies. The cells were incubated with the

antibodies for 30 mins at 4°C, and then the plate was centrifuged for 5 mins at 200g in a Sorval RT7plus centrifuge. Buffer containing excess unbound antibody was removed and cells were washed by resuspension in 200µl fresh FACS buffer per well followed by centrifugation as before. Cells were then fixed by resuspension in FACSFix buffer (PBS with 2% paraformaldehyde). Staining was analyzed on a FACSCalibur® (Becton-Dickinson U.K. Ltd. Cowley, Oxford, UK) flow cytometer using CellQuest software (Becton Dickinson U.K. Ltd.).

2.7.2.3 Isolation of murine splenic dendritic cell populations for RNA extraction

Dendritic cells were isolated as described above. After enrichment with $CD11c^+$ MACS beads, the positively selected cells were stained with CD11c-APC, B220-FITC, and PE-conjugated CD3, CD19 and DX5 (0.5µl antibody stock per 10^6 cells) for 30 min on ice. Cells were centrifuged for 5 mins at 200g and unbound antibody in the supernatant was removed. Fresh buffer was added to wash the cells and the resuspended cells were pelleted again by centrifugation. Resuspended cells were sorted using a Mo-Flo cell sorter (Cytomation, Fort Collins, Colorado USA), isolating $CD11c^+$ $B220^+$ and $CD11c^+$ $B220^-$ populations with $CD11c^+$ PE^+ cells excluded. RNA was extracted from a equal number of cells of each subpopulation in every sample in each specific experiment, typically between $2x10^4$ and $1x10^5$ cells.

2.7.2.4 Analysis of cell surface markers expression on splenic $CD11c^+$ DC subpopulations from persistently infected mice

After positive selection as above, $CD11c^+$ cells were resuspended in FACS buffer and cells expressing non-DC lineage were depleted. A cocktail of biotinylated depletion antibodies were added at a concentration of 1µl antibody per 10^6 purified

cells. Antibodies used for depletion were biotinylated anti-CD3, anti-CD19 and anti-DX5. Cells were incubated with the antibodies for 30 mins at 4°C before excess antibody was removed by pelleting the cells by centrifugation for 5 min at 1500rpm and removing the unbound antibody-containing supernatant. The cells were washed by resuspension in fresh buffer followed by centrifugation at 200g for 5 mins. Spent buffer was removed and the cells were resuspended in buffer containing CELLection Biotin-Binder streptavidin-conjugated magnetic Dynal beads added at a concentration of 250µl beads per 10^8 cells. Beads and cells were incubated on a rotating incubator (Jencons) at 4°C for 20 mins, then excess beads were removed and the cells were washed by centrifugation as before. After resuspension in fresh FACS buffer, bound $CD3^+$, $CD19^+$, $DX5^+$ cells were depleted by magnetic separation, which was performed twice for each sample, leaving unbound cells in the supernatant which were washed as before. Depleted cells in FACS buffer were dispensed into 96-well V-bottomed plates at a concentration of $1x10^5$ cells per well in 100µl and stained with PE-conjugated antibodies to MHC class I ($H\text{-}2K^b$), MHC class II (I-A/I-E), CD40, CD80 (B7.1), CD86 (B7.2) or CD54 (ICAM-1). The cells were co-stained with FITC-conjugated anti-PDCA-1 and APC-conjugated anti-CD11c to identify plasmacytoid DCs, or with FITC-conjugated anti-CD4 and APC-conjugated anti-CD11c to identify $CD4^+$ DCs or alternatively with FITC-conjugated anti-CD8α and APC-conjugated anti-CD11c to identify $CD8^+$ DCs. Other cells were also stained with appropriate isotype control antibodies. The cells and mAbs were incubated in the dark at 4°C for 30 mins, then the cells were washed as described previously and resuspended in FACSFix buffer. Data was collected on a FACSCalibur® (Becton-Dickinson) flow cytometer and analysed using CellQuest software (Becton Dickinson).

The mean fluorescence intensity (MFI) of surface marker staining on DCs of each subset was calculated as the MFI of cells stained with marker-specific mAb minus the MFI of cells stained with an isotype-matched control mAb.

2.7.3 Stimulation of CD11c$^+$ DCs with TLR ligands

CD11c$^+$ cells isolated from murine spleens as described in section 2.7.2.1 were seeded at 1x10^5 cells per well in a 96-well U-bottomed plate in 100µl of medium containing one of the following: polyIC (100µg/ml, 10µg/ml or 1µg/ml), R848 (100µg/ml, 10µg/ml or 1µg/ml), CpG (2nmole/ml, 0.2nmole/ml or 0.02nmole/ml) or LPS (100ng/ml, 10ng/ml or 1ng/ml). After 24 hours stimulation at 37°C, the plate was centrifuged at 1200rpm for 5 mins and the supernatant collected and stored at -80°C prior to analysis of its cytokine content.

2.7.4 Confocal studies on spleen sections from mice acutely or persistently infected with LCMV Arm or Cl13

2.7.4.1 Purification and labelling of mouse anti-NP113 mAb

Hybridoma supernatant from large scale cultures of murine anti-NP113 mAb hybridoma cells was prepared by Julie Lewis and stored at -20°C until purification. Antibody was purified on a Protein A affinity column following the manufacturer's instructions. The antibody was eluted in amine-containing elution buffer at pH 2.8, which was neutralized by the addition of 1M Tris buffer at pH 9. Fractions were collected, and the amount of protein in each fraction was determined by carrying out UV spectroscopy at 260nm, 280mn and 320nm using the Christian and Warburg equation to calculate the protein content. The fractions containing the highest amounts of protein were pooled and dialyzed in Slide-A-Lyzer® dialysis cassette with a

molecular cut-off of 10000 MW against PBS. After buffer exchange, the antibody was again quantitated by UV spectroscopy before it was concentrated to a concentration of 1mg/ml in PBS on a Microcon-YM100 centrifugal filter device with a 100 000 MW molecular weight cut-off by centrifugation at 14,000g on an Eppendorf 5417R fixed rotor centrifuge (Eppendorf, Hamburg, Germany) according to manufacturer's instructions.

1mg of purified antibody was conjugated to Cy-5 using a Cy-5 labelling kit, following the manufacturer's instructions. Briefly, the purified, dialyzed antibody was allowed to react with Cy-5 dye to a enable conjugation to the carbohydrate moieties on the Fc portion of the antibody. Unbound dye was separated from the labelled antibody by size-exclusion column chromatography. Purified, labelled antibody was quantitated by uv spectroscopy (concentration at 0.1μg/ml) and stored at 4 °C.

2.7.4.2 Staining protocol to visualize IFN-α, pDC and LCMV-NP protein on spleen sections

Pieces of spleen were embedded in O.C.T compound (<11% Polyvinyl alcohol, <5% Carbowax) in plastic tissue moulds and stored at -20°C. Cryosections of 5-8 μm were cut on the Leica CM1900 cryostat (Leica Instruments, Nussloch, Germany), transferred onto poly-L-lysine slides and fixed with acetone for 10mins at 4°C. The sections were rehydrated with PBS and blocked with a 1:1 mix of 1x blocking reagent from a tyramide signal amplification kit and 1x M.O.M. mouse Ig blocking reagent (90μl reagent per 2.5ml PBS) for 1 hour at RT. Rat anti-IFN-α diluted 1 in 2 and mouse anti-NP113-Cy5 diluted 1/10 in M.O.M. diluent (600μl protein concentrate solution diluted into 7.5ml PBS) were then added. After incubation for 2 hours at RT, slides were washed 3x with PBS for 5 min per wash. Goat anti-rat AlexaFluor 488

diluted 1/50 was added for 1 hour at room temperature (RT) in a humidified chamber. Excess antibody was removed and slides were again washed 3x with PBS and then blocked for 30mins with 5% normal rat serum. Biotinylated rat-anti-m-PDCA-1 diluted 1/5 in tyramide blocking buffer was added and the slides were incubated overnight at 4°C. After washing 3x in PBS as before, streptavidin –HRP diluted 1/100 in tyramide blocking buffer was added and incubated for 1 hour at RT. Slides were washed as before then 1x tyramide-Alexa Fluor 594 substrate solution was added and allowed to react for 7 mins. After washing 3x, with a final 10 min wash in PBS, the slides were mounted with one drop of Vectashield fluorescent preservative reagent prior to visualization by confocal microscopy. To ensure specificity of labelling, sections were stained in parallel with appropriate isotype control antibodies, namely, unconjugated rat-IgG1 (0.5mg/ml) at 1/5 dilution, biotin-rat-IgG at 1/25 dilution and mouse-IgG2a-Cy5 diluted 1/10 (all isotypes from. The results were recorded using software for the Leica TCS NT confocal system (Leica, Knowlhill, UK). Staining for IFN-α appeared green, LCMV-NP staining appeared blue and pDCA-1 staining appeared red.

2.7.5 Analysis of the response of mice persistently infected with LCMV Cl13 to innate stimuli

At 3 weeks post infection, LCMV Cl13 infected mice and uninfected controls were stimulated with intra-peritoneal (i.p) injections of one of the following TLR stimuli in 200µl PBS: 5µg polyIC, 50 nmoles R848, 25 nmoles CpG 1826 or with 800 haeagglutinin (H.A) units of influenza virus PR8. Mice were culled at timepoints between 6 hours and 24 hours post-stimulation and blood and tissues removed as described in section 2.7.1. For all experiments, 3 mice per treatment type and 1

uninfected control were culled at every timepoint from both the LCMV-infected and uninfected groups.

2.7.6 Detection of cytokines by cytokine bead array (CBA) assay

A murine inflammation CBA kit was used to measure the levels of IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12 p70 in the serum of mice exposed to different innate stimuli. In CBA assays, capture beads which have discrete fluorescent intensities bind to specific cytokines and these cytokines are further detected using PE-conjugated mAbs specific to the cytokines.

CBA assays were carried out following the manufacturer's instructions. Supernatants were thawed at 4°C just before the start of the assay. Serial dilutions of the standards were prepared in assay diluent to cover concentrations from 20-5000 pg/ml of each cytokine measured. Master-mixes of the capture beads were prepared by mixing 6µl of each cytokine capture bead per sample to be tested. 35µl of diluted serum (diluted between 1:2 and 1:8 in assay diluent to cover the range of cytokines measured) or the dilutions of the standards were mixed with 35µl of the capture bead master-mix and 35µl of the detection reagent containing PE-conjugated anti-mouse cytokine mAbs. The mix was incubated for 2 hours at RT in the dark. Samples were washed in 1ml of wash buffer and centrifuged at 200g for 5 mins in a Sorvall RT7 centrifuge. The supernatant was discarded and beads resuspended in 300µl FACSFix buffer to inactivate any LCMV in the sample prior to flow cytometric analysis. Cytokine levels were then assessed using a FACSCalibur™ flow cytometer with FASComp software (Becton Dickinson) for instrument settings. The data was analysed using CellQuest™ software.

2.8 Analysis of Type 1 IFN production during *in vitro* persistent LCMV infection

2.8.1 Production and infection of murine bone marrow derived-DC (BM-DC)

Hind limbs were collected from C57BL/6 mice and tibia and femurs were dissected using forceps and scissors. After removing the flesh, the bones were cleaned by spraying with 70% ethanol and placed in complete DC medium (RPMI supplemented with 10% (v/v) FCS, 1μg/ml P/S, 100U/ml polymyxin B and 50mM β-mercaptoethanol). The ends of the bones were cut off to create a hollow tube. The marrow was flushed out of the bones using a 27G needle attached to a syringe containing complete medium. This was repeated until all the marrow was removed, then the marrow was passed through the syringe to generate a single cell suspension. The number of bone marrow precursor cells, which are large, irregular cells, in the suspension was counted, and the cell concentration adjusted to $2x10^5$/ml in complete DC medium supplemented with 20ng/ml mouse GM-CSF. The suspension was plated into 100x150 mm Petri dishes at 10ml per dish and incubated at 37°C in 5% $C0_2$ for 8 days to allow bone marrow precursors to differentiate into DCs. At day 3 of culture, the dishes were gently swirled and an extra 10ml of complete RPMI medium supplemented with 20ng/ml of murine GM-CSF was added into each dish. At day 6, 10ml of spent medium was removed and centrifuged to pellet the cells therein. The cell pellet was resuspended in 10ml of fresh complete RPMI medium, again supplemented with 20ng/ml of murine GM-CSF and returned to the dish. Cells were harvested at day 8 of culture. The medium was removed and collected in 50ml centrifuge tubes, then 5ml of accutase was added to each dish. This was incubated briefly in a 37°C 5% CO_2 incubater, then the dishes were swirled about to dislodge the adherent cells which was transferred to the centrifuge tubes. Fresh accutase was

added to the dish, the incubation and swirling repeated until all of the adherent cells were removed.

Harvested cells were centrifuged and resuspended in fresh media. The yield was calculated and the cells divided into 15ml centrifuge tubes at a concentration of $3.2x10^7$ cells per tube. The cells were pelleted by centrifugation and then infected with LCMV Arm or clone 13 at appropriate m.o.i. Virus was diluted in serum-free RPMI or PBS to a volume of 0.5-1ml, and the tubes were flicked to dislodge the cell pellet and mix the cells with the viral suspension. The tubes were incubated in a 37°C, 5% CO_2 incubator for 1 hour to allow viral attachment and entry. Tubes were then centrifuged, all the viral suspension removed and the cells were resuspended in fresh complete medium and plated out into 35mm x 10mm Petri dishes at $2x10^6$ cells per dish in 1ml complete media supplemented with 20ng/ml GM-CSF.

2.8.2 Generation of cell lines chronically infected with LCMV Arm and Cl13

MC57, BalbC17, FSDC or D2SC/1 cells or BM-DC were seeded into 6-well plates at $1.5x10^5$ cells per well or $1x10^6$ cells/well in 60mm dishes in 1 ml medium and incubated overnight. The supernatant was then removed and the cells were infected with LCMV Arm or LCMV Cl13 at a m.o.i of either 0.1, 0.5, 5 or 10 in 500µl serum-free medium for 1 hour at 37°C. After the incubation, the virus containing medium was removed and the cells were washed with PBS to remove excess virus. Wash buffer was removed by centrifugation at 300g for 5 mins. Infected cells were overlaid with 3ml growth media and cells and supernatant were sampled at indicated timepoints. Cells were transferred into small flasks when they appeared confluent, usually at 48 or 72 hours and were then passaged twice a week. Plaque assays (section

2.3.2) and immunostaining (described in the following section) were performed to confirm that the cells were chronically infected with LCMV.

2.8.3 Immunostaining of chronically infected cells

At various timepoints post-infection, cells were harvested and resuspended in PBS at a concentration of ~5 x 10^6 cells/ml. 50µl of the cell suspension was spotted onto wells of Teflon slides. The remaining liquid was sucked off after 2-3 mins and slides were left to dry under the hood for about 1 hour. Slides were fixed with 100% methanol at -20°C for 10 mins and allowed to dry. Fixed cells were rehydrated with 50µl of PBS-2% BSA for 30 mins. For infected DC cells (FSDC, D2SC/1 or BM-DC), Fc-mediated non-specific binding was blocked by treatment with the M.O.M. kit. The buffer was then removed, 20µl of anti-LCMV ascites VR164 at a 1/50 dilution was added and the slides were incubated for 1 hour at 37°C in a humidified incubator. Slides were washed by immersion in PBS for 5 min. 20µl of FITC-conjugated goat anti-mouse IgG and IgM (H+L) diluted 1/200 was added. After incubation for 50 mins at room temperature, the slides were washed by immersion in PBS for 3 mins. Bo-Pro diluted 1/200 in blocking buffer was added to visualize the nuclei. After 15 min incubation at room temperature, the slides were washed 3x by immersion in PBS for 3 mins, with a final immersion for 15 mins. A drop of Vectashield was added on to each slide prior to visualization. LCMV infected-cells were visualised and the images captured by confocal microscopy (Leica Microsystems (UK) Ltd, Milton Keynes, UK). Viral antigen appeared green and the nuclei appeared red.

2.8.4 Generation of stably transfected cells expressing LCMV proteins

2.8.4.1 Transfection of individual LCMV genes into FSDC and D2SC/1 cells

FSDC and D2SC/1 cells were transfected with plasmids encoding individual LCMV proteins described in section 2.4.3. Cells were seeded into 6 well plates at $1x10^5$ cells per well. Effectene Transfection Reagent (Qiagen) at the ratios below was used to transfect each LCMV plasmid into FSDC or D2SC/1 cells. LCMV plasmids were co-transfected with pcDNA6 V5-HisB at a ratio of 5:1:

FSDC 1μg DNA in 0.5μl vol: 10μl Condensation buffer :10μl Effectene in 1ml media

D2SC/1 1μg DNA in 0.5μl vol: 8μl Condensation buffer :50μl Effectene in 1ml media

Cells were incubated in transfection reagent overnight, then this was replaced with complete growth medium. After 24 hours incubation in a 37°C, 5% CO_2 incubator, the medium was replaced with selection medium, which was complete growth medium supplemented with 10ug/ml blasticidin. Cells were cultured for a week in selection medium, during which time cells expressing the blasticidin resistence genes survived and multiplied. The survivors were resuspended with accutase and cloned by limiting dilution into 96-well plates. Single cell colonies were identified and the cells tested for expression of the respective LCMV protein by immunofluorescence.

2.8.4.2 Selection of positive colonies by immunofluorescence

Single-cell colonies which survived in the selection media were expanded from 96-well to 24-well plates. Some of the cells transferred onto wells of a Teflon slide and fixed and stained for LCMV protein expression using the method described in

section2.8.3. Colonies that were strongly positive for GP or NP expression were selected and subsequently expanded into clonal cell lines. Antibody specific for Z protein was not available, and for these cells, blasticidin positive colonies were cloned and one clone was selected and used in subsequent experiments.

2.8.5 Stimulation of infected cells with TLR ligands

BalbCl7, D2SC/1 or FSDC cells infected with LCMV Cl13 and their respective uninfected controls were seeded at $1x10^5$ cells/well in 100µl of medium. After an overnight incubation, supernatant was replaced with media containing the following TLR ligands:

PolyIC at 100µg/ml, R848 at 50µg/ml, CpG 1826 at 2nmole/ml or medium only as a control. The cells were incubated overnight at 37°C for 24 hours. The plates were then centrifuged at 200g for 5 mins, and the supernatant was collected and stored at -80°C until tested. Cell pellets were lysed with RLT buffer and RNA isolated using the RNeasy Mini kit (or were stored at -80°C until RNA extraction).

2.8.6 Infection of LCMV Cl13-infected or transfected cells with Sendai or HSV

Uninfected cells and cells infected 80 days previously with LCMV Cl13 were seeded at $1x10^5$ cells per well in U-bottomed 96-well plates. After an overnight incubation, cells from one well was resuspended and counted, and cells were infected with Sendai virus at 1 or 10 m.o.i. or HSV at an m.o.i. of 5, diluted in PBS. After 1 hour incubation at 37°C, the virus was removed and replaced with growth medium. At 4 and 18 hours post-infection, the plates were centrifuged at 200g for 5 mins in a Sorvall RT7 centrifuge and cells and supernatant were collected as described in section 2.8.5 and stored at -80°C prior to analysis.

2.9 Statistical Analysis

Statistical analyses were performed on Minitab Release 14 software (Minitab Ltd, Coventry, UK). The significance of differences in the levels of type 1 IFN produced by uninfected and persistently infected ex vivo $CD11^+$ DCs upon stimulation with TLR ligands was addressed by 1-sample t-test. Analysis of Variance (ANOVA) using a general linear model with Tukey's pairwise comparison was used to determine the statistical significance of the following:

1. The difference in the total numbers of DCs in the spleens of uninfected versus persistently infected mice
2. The difference in the levels of type 1 IFN induced in uninfected and persistently infected mice upon exposure to innate stimuli
3. The difference in the maximal levels of type 1 IFN produced by cultured cell lines in response to infection with different RNA viruses or stimulation with polyIC.
4. The difference in the levels of type 1 IFN produced by uninfected and persistently infected cell lines in response to stimulation with TLR ligands
5. The difference in the levels of type 1 mRNA transcript produced by uninfected and persistently-infected cell lines upon Sendai or HSV viral infection
6. The difference in the levels of type 1 IFN activity produced by uninfected and persistently infected cell lines upon Sendai or HSV viral infection

CHAPTER 3

DEVELOPMENT OF SENSITIVE ASSAYS FOR THE DETECTION OF MURINE TYPE 1 IFNs AT THE PROTEIN AND mRNA LEVELS

3.1 Introduction

Murine type 1 IFNs were first described in 1961 by their resistance to acid wash at pH 2.0 and their ability to protect L929 cells from infection with EMCV (Hitchcock and Porterfield 1961). Methods to detect type 1 IFNs have advanced since then and it now possible to visualise type 1 IFN protein *in situ* and to quantitate type 1 IFN by bioactivity, protein concentration or by the relative abundance of type 1 IFN mRNA within cells (Deonarain et al. 2000; Lewis 1995; Meager 2002). Some of the methods currently in use are discussed below.

The purpose of the series of experiments described in this chapter was to design and optimize sensitive means to measure type 1 IFN produced by cultured cells or in mouse tissue or serum. Existing methods for quantitation of type 1 IFN activity or protein were evaluated for their sensitivity and ease of application on large sample numbers. In addition, efforts were made to develop new, more sensitive methods for quantitation of type 1 IFNs at the protein and mRNA level. The most appropriate assays were then selected for application in subsequent *in vivo* and *in vitro* studies.

3.1.1 IFN-α ELISA

A kit to measure murine IFN-α is commercially available from PBL Laboratories, and several labs have developed type 1 IFN sandwich ELISAs using commercially available anti-murine IFN-α antibodies (Asselin-Paturel et al. 2001; Diebold et al. 2003). Monoclonal antibodies against IFN-β were not commercially available at the

time this project was initiated, but an ELISA kit to measure murine IFN-β has since been introduced (RnD Systems, Abingdon, UK). Samples, which can be either supernatants or serum, are quantitated against a standard curve of known IFN-α concentration. While ELISAs are useful in that they provide partial information regarding the type of IFN secreted, they are relatively insensitive, with the detection limit of commercial IFN-α murine ELISAs reported to be ~10 pg/ml (Murine IFN-α kit technical notes (2004)).

The commercial IFN-α ELISA purports to recognise IFN-αA,-α1, -α4, α5, α6 and α9 subtypes while the number of subtypes recognized by the laboratory-developed sandwich ELISA is unclear. At the start of the project, the PBL ELISA from the kit and a laboratory-developed protocol (Diebold et al. 2003) were evaluated, so that their limits of sensitivity could be compared to those of other methods.

3.1.2 Antiviral bioassay

The antiviral assay, first developed in 1981 by Familletti *et al* (Familletti et al. 1981) to quantitate human type 1 IFN, is the "gold standard" for measurement of type 1 IFN activity. It measures the antiviral bioactivity of IFN-α, IFN-β and to a lesser extent IFN-γ in a given sample based on their ability to protect a monolayer of cells from lysis by vesicular stomatitis virus (VSV) or EMCV. To assay murine type 1 IFN activity, L929 cells at a fixed concentration of $1x10^5$ cells/well are seeded in wells in a 96-well plate containing serial dilutions of an IFN standard and the test sample. IFNs in the standards and sample signal through the IFN-α/β and γ receptors on the cell surface to activate the expression of anti-viral genes, and establish an anti-viral state within the cells. 24 hours later, the standard and sample are removed and replaced with medium containing the lytic viruses VSV or EMCV at a m.o.i of 0.1. If

an anti-viral state has been achieved, the monolayer will be resistant to infection and will appear intact when stained 16 to 24 hours later with crystal violet. The amount of type 1 IFN that protects 50% of the monolayer from lysis is defined as 1U of IFN. As such, the limit of detection for this assay is 1U of IFN activity. The total amount of type 1 IFN in a given sample can then be calculated by multiplying the dilution at which there is 1U of IFN in the sample with the dilution factor. In practice, a standard curve of known type 1 IFN activity is run on every plate as reference. The activity of type 1 IFN in cell culture supernatant, serum and homogenized tissue samples can be assayed using this method.

Although the viral protection bioassay provides a very sensitive indirect measurement of type 1 IFN activity, it does not provide any information on protein concentrations. Another drawback of this assay is that is does not distinguish between the activity of IFN-β or IFN-α unless saturating amounts of IFN-subtype specific neutralizing antibodies are added; neither can it give information on the distinct IFN-α subtypes present in a sample.

3.1.3 Reporter gene-based bioassays

The expression of antiviral proteins such as Mx is induced by the presence of type 1 IFNs as their genes contain promoter elements that are recognized by transcription factors which are activated via the type 1 IFN signalling cascade initiated by the binding of type 1 IFNs to its receptor (Platanias 2005). New cell-based assays to quantitate type 1 IFN activity have exploited the specificity of these promoter elements by linking them to a promoter-less reporter gene such as luciferase, secreted alkaline phosphotase or human growth hormone on an eukaryotic expression plasmid (reviewed in (Meager 2002)). These plasmids are transfected into cells, which are

stimulated with type 1 IFNs. The level of reporter protein that accumulates within the cells is proportional to the quantity of the type 1 IFN stimulus in a linear dose-response relationship. Such reporter assay systems offer a means to quantitate IFN activity in a manner that is more direct and specific than the traditional antiviral bioassays, and with a quicker assay time. Reporter assays to measure IFN from several species have been described (Canosi et al. 1996; Fray et al. 2001; Hammerling et al. 1998; Lleonart et al. 1990), including an assay to quantitate the activity of murine IFN-γ (Lewis 1995). The sensitivities of reporter gene-based assays varied from system to system. Some systems, such as assays established for the detection of bovine type 1 IFNs, are reported to respond to as little as of 0.32 U/ml of type 1 IFN and posses a dose-dependent range of up to 125 U/ml (Baigent et al. 2002; Fray et al. 2001), which was more sensitive than the bioassay. However, like the bioassay, current assays are not able to distinguish between the activity of IFN-β and IFN-α or different IFN-α subtypes. One of the aims of the project was to develop a reporter gene-based assay to measure type 1 IFNs from murine samples. In 2005 an assay to measure type 1 IFN on the basis of percentage of cell expressing the eGFP reporter gene was developed (Bollati-Fogolin and Muller 2005). Murine embryonic fibroblast (MEF) cells were immortalized from mice genetically engineered to contain Cre-recombinase linked to an Mx promoter, and eGFP gene containing a loxP flank stop cassette silencer element. Upon stimulation with type 1 IFN, expression of cre-recombinase was induced, which when expressed, deleted the loxP silencer element, allowing expression of eGFP. This system was reported to have a detection range of between 20-300U and 10-500U for IFN-α and IFN-β respectively, which is less sensitive than the bioassay; and like the bioassay, current reporter assays are not able to distinguish between the activity of IFN-β and IFN-α or different IFN-α subtypes.

3.1.4 Detection of specific type 1 IFN subtype mRNA by RT-PCR

It would be of interest to be able to dissect the responses of individual subtypes of type 1 IFN, as evidence exists that they may be differentially regulated during the response to viral infections. This is difficult to perform using bioassays - while it is possible to dissect the IFN-β response from the IFN-α by addition of neutralizing antibodies, dissecting the individual contributions of the different IFN-α subtypes is not yet possible as IFN-α subtype-specific neutralizing antibodies are not available. For this purpose, RT-PCR assays have been developed (Deonarain et al. 2000; Fung et al. 2004). PCR primers specific for IFN-β, IFN-α4 and all IFN-α subtypes have been designed making it possible to measure induction of individual type 1 IFN types and subtypes. Such assays are also particularly suited to quantitating type 1 IFN responses in cells or tissues, in contrast to bioassays which are useful to measure type 1 IFN activity in serum or supernatant. Total or mRNA is purified from cells or tissues and used as template RNA in PCRs using the specific primer to amplify distinctive subtypes. When the template is also tested with a housekeeping control PCR, the assay becomes semi-quantitative as the amplified products are electrophoresed and detected on an agarose gel and then individual bands quantitated by densitometric analysis. An obvious limitation of the RT-PCR assay is that it can not measure type 1 IFN levels in serum or supernatant samples. Although RT-PCR will not give information on protein concentration or bioactivity, its key advantage over the other methods is that it is a fast, direct method that enables dissection of the different subtypes of type 1 IFN induced under various conditions in samples that can not be readily analysed using cellular or ELISA assays. However as reaction products of the RT-PCR assay are only detected at the endpoint of a PCR reaction, they only

provide qualitative or at best semi-quantitative results regarding the presence and levels of various IFN types or subtypes in a given sample.

Real-time quantitative reverse transcription PCR (RT-PCR) is a recent innovation in the field of analysis by PCR. In a modification of the conventional PCR assay, primers and probes that specifically recognize a region within a given gene of interest are designed that can be run under universal thermal cycling conditions. The probe, which binds a sequence in between the forward and reverse primers, is degraded during the extension phase of the PCR amplification and releases a fluorescent signal; the amount of fluorescence released is directly proportional to the amount of product generated in each PCR cycle and acts as a quantitative measure of PCR product formation. The technology is well-suited for high throughput analysis – assays are conducted in a 96-well plate format and direct monitoring of the fluorescent signal during PCR amplification does away with the requirement for post-PCR analysis. Since its introduction, assays to measure a whole range of murine and human cytokines, chemokines and immune-related factors have been designed (Overbergh et al. 1999). Real-time quantitative RT-PCR primers and probes that detect murine IFN-β and IFN-α4 had been published soon after the project was initiated (Karaghiosoff et al. 2003), and reagents for quantitating distinct human IFN-α subtypes using this approach had been developed (Loseke et al. 2003). It was planned to design and optimize additional real-time quantitative RT-PCRs assays that recognized either all murine IFN-α subtypes or distinct IFN-α subtypes to complement the existing IFN-β and IFN-α4 assays, to enable comprehensive analysis of the type 1 IFN response in cells or tissues during acute and persistent LCMV infection.

In summary, at the time of the initiation of this project, a number of systems existed to measure type 1 IFN protein levels in biological samples. Each system has its strengths, weaknesses and particular utility as summarized in the next table.

Table 3.1 Summary of aspects of various IFN-α/β quantitation methods

Assay	Quantitates	Advantages	Disadvantages
ELISAs	IFN-α protein concentration IFN-β protein concentration	Direct, simple Able to distinguish IFN-α from IFN-β levels	Relatively insensitive No ELISAs are available for IFN-α subtypes
Antiviral bioassay	IFN-α/β activity	Very sensitive assay	Indirect, relatively laborious assay Doesn't distinguish between IFN-α and IFN-β Unclear whether all subtypes show equal activity in this assay
Reporter gene-based bioassay	IFN-α/β activity	Simpler than the antiviral bioassay Potential for high sensitivity	Indirect assay Does not distinguish between type 1 IFN subtypes Again, unclear if all subtypes show equal activity in this system
Quantitative RT-PCR	IFN-α or -β mRNA levels	Able to distinguish between IFN subtypes Very sensitive	Only suitable for use in cells/tissues mRNA levels may not correspond to protein or bioactivity levels.

This chapter describes the steps that were taken to evaluate the existing assays and also develop new, potentially more sensitive assays for detection of murine type 1 IFN bioactivity. The aims were to

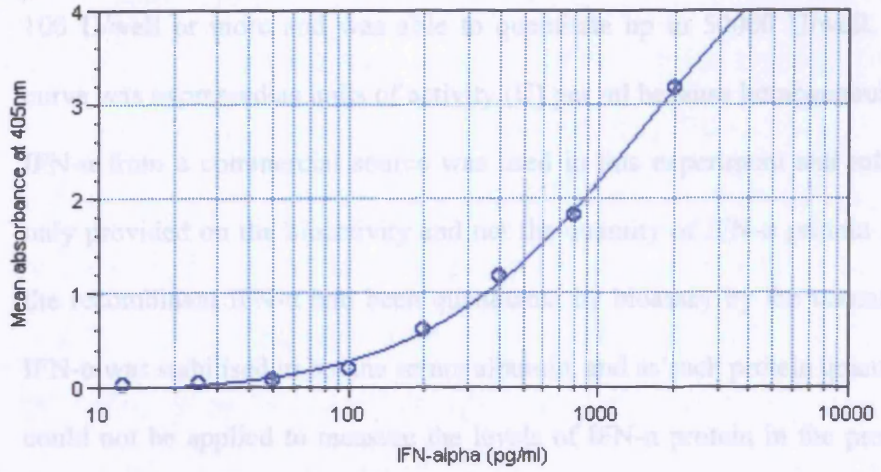
1) Perform IFN-α ELISAs and EMCV-based antiviral bioassays in order to evaluate the level of sensitivity of each
2) Set up reporter gene-based assays for the detection of murine type 1 IFN bioactivity, and compare their performance to that of the IFN-α ELISA and the antiviral bioassay
3) Establish quantitative RT-PCR assays for detection of IFN-β, all IFN-α subtypes and selected IFN-α subtypes, and evaluate their performance.

3.2 Quantitation of IFN-α protein levels by IFN-α ELISA

In order to determine the sensitivity with which IFN-α was detect by the commercially available IFN-α ELISA, an assay was conducted testing dilutions of a series of concentrations of IFN-α that was supplied by the manufacturer. Figure 3.1A depicts a standard curve generated from the known concentrations of recombinant IFN-α when the IFN-α sandwich ELISA was conducted according to the steps described in section 2.5.1. This ELISA appears to detect IFN-α only when it is in concentrations of more than 50-100 pg/ml. The concentrations used in the experiment did not achieve saturation, and so range of detection appears to be up to more than 2000 pg/ml, which was the highest concentration tested.

Next the laboratory-based IFN-α ELISA protocol was run to determine whether this provided more sensitive detection of IFN-α. Figure 3.1B shows the standard curve generated. This ELISA was able to detect IFN-α only when it was in concentrations of

A)



B)

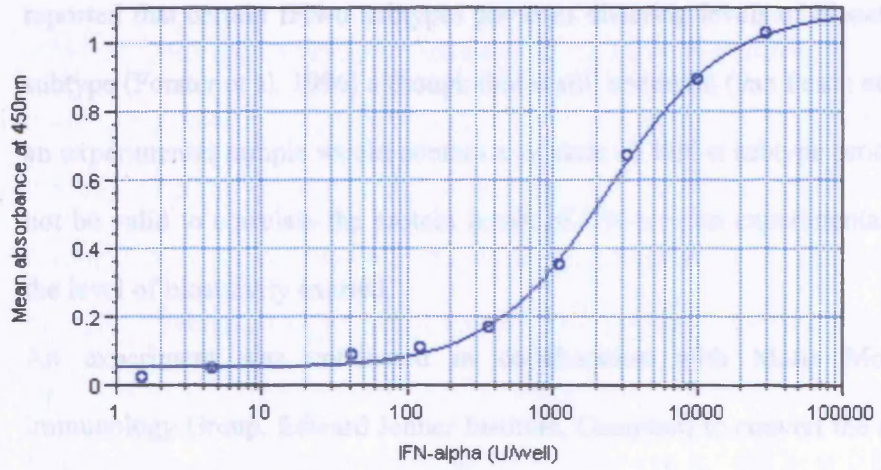


Figure 3.1 IFN-α ELISA standard curves generated using the commercially-available kit and the lab-developed assay

The sensitivity of IFN-α sandwich ELISAs carried out using the commercially available kit (A) or the laboratory-developed protocol (B) were assessed by assaying known concentrations of recombinant IFN-α, serially diluted to cover a range from 0.15 to 2000 pg/ml (A) or 1 to 50000 U/well (B). The ELISA results, which were the mean absorbance at 405nm from duplicate wells for the commercial protocol and at 450nm from triplicate wells for the laboratory-based protocol, were plotted against the sample concentration to obtain at standard curve. A representative standard curve for each assay generated by the SOFTMax Pro Analysis software is shown.

100 U/well or more and was able to quantitate up to 50000 U/well. The standard curve was expressed as units of activity (U) per ml because homogenous recombinant IFN-α from a commercial source was used in this experiment and information was only provided on the bioactivity and not the quantity of IFN-α protein in the prep as the recombinant IFN-α had been quantitated by bioassay by the manufacturers. The IFN-α was stabilised in bovine serum albumin, and as such protein quantitation assays could not be applied to measure the levels of IFN-α protein in the prep. ELISAs in fact quantitate IFN protein and not activity levels. Neither the commercial nor the laboratory-developed ELISAs has been shown to detect all α subtypes and it has been reported that certain IFN-α subtypes posseses differing levels of bioactivity to other subtype (Forster et al. 1996) although this is still uncertain (van Pesch et al. 2004). As an experimental sample would contain a mixture of IFN-α subtype proteins, it would not be valid to correlate the protein levels of IFN-α in an experimental sample with the level of bioactivity exerted.

An experiment was conducted in collaboration with Maria Montoya (Viral Immunology Group, Edward Jenner Institute, Compton) to convert the activity of the recombinant IFN-α into protein concentrations. Standards of known activity were tested in the commercial ELISA alongside the standard curve of IFN-α protein concentration. 35 U/ml of IFN-α from the laboratory-based assay corresponded to 1pg/ml of IFN-α.

Thus, the sensitivity of the laboratory-based ELISA was ~2.8 pg/ml, with a range of up to1428 pg/ml, which indicated the laboratory-based ELISA was more sensitive and possibly possessed a wider range of IFN-α subtype detection than the commercially available ELISA.

3.3 EMCV Antiviral protection bioassay

To evaluate the specificity and sensitivity of the antiviral bioassay, L929 cells were treated with 2-fold serial dilutions of recombinant IFN-α, IFN-β or IFN-γ in the presence or absence of neutralizing anti-IFN-α, -β or –γ antibodies prior to infection with EMCV. Figures 3.2 shows images of the results obtained. As anticipated, IFN-α, -β and –γ protected the L929 monolayer from CPE mediated by EMCV infection. For IFN-α and –β the visual endpoint, which was the transition from a monolayer which was completely intact to a monolayer with CPE, occurred when the standards were diluted to 2 $\log_2$ (3.9)

U/ml. For IFN-γ the visual endpoint of the assay occurred at 1$\log_2$ (2)U/ml. The difference between the expected endpoint of 1U/ml and observed endpoint of 4 U/ml may be the result of differences in the method employed to titrate the IFN, such as the use of different viruses (the potency of recombinant IFN-α, IFN-β and IFN-γ was determined by the manufacturer by viral inhibition bioassay but the virus employed was not disclosed), or the sensitivity of the particular strain of L929 cells to the EMCV virus or the fact that the standards may have lost some of their activity during the freezing and thawing process – the standards were derived from aliquots of stock recombinant IFN-α, -β or –γ that had been distributed into small aliquots and stored frozen at -20°C prior to use.

Addition of neutralizing antibodies against IFN-α abolished the protection conferred by this cytokine, although there was some residual activity observed at the top concentrations of 10 $\log_2$ (500) and 9 $\log_2$ (250)U/ml of IFN-α, despite addition of neutralizing antibodies at a ratio of 2 neutralizing units to 1 unit of IFN-α. This residual protection was also observed when neutralizing antibodies against IFN-β were tested at similar ratios against high concentrations of IFN-β.

The bioassay was to be used on samples which would contain a mixture of subtypes of type 1 IFN. In order to demonstrate the specificity of the neutralizing anti-IFN-α and IFN-β antibodies in such samples, the neutralizing antibodies were tested in a mixture of recombinant IFN-α and β, combined in a 1:1 ratio starting at a top concentration of 100U/ml (Figure 3.2). When pre-incubated with either neutralizing anti-IFN-α or anti-IFN-β polyclonal antibody at a ratio of 2 neutralizing units to 1 U of IFN, the endpoint occurred 2 wells (or 4 dilutions) earlier in the presence of neutralizing antibody than in wells without neutralizing antibody, indicating that the concentrations of bioactive type 1 IFNs the wells with neutralizing antibody had halved, consistent with specific neutralization of only one of the two species of IFN in the sample.

It was anticipated that the samples tested in the bioassay will contain LCMV, which could potentially infect the L929 monolayer. LCMV does not lyse L929 cells as it instead establishes a persistent infection in these cells (Bruns et al. 1988; Gessner and Lother 1989; Weber et al. 1983); however, it was unclear whether these infected L929 cells produce type 1 IFNs in response to the infection. Therefore, the capacity of L929 cells to produce endogenous type 1 IFN following exposure to samples containing LCMV virus or residual TLR ligands from *in vitro* stimulation experiments was investigated. Figure 3.3 shows the result of an experiment in which L929 cells were treated with serial 2-fold dilutions of LCMV starting at 10^6 p.f.u./100μl media/well. A standard curve of 2-fold serial dilutions of recombinant IFN-α (Hycult), with a top concentration of 500 U/ml was set up in parallel. Based on the results shown in Figure 3.3, L929 cells do not produce detectable levels of endogenous type 1 IFN when exposed to up to 10^6 p.f.u./100μl media/well of LCMV.

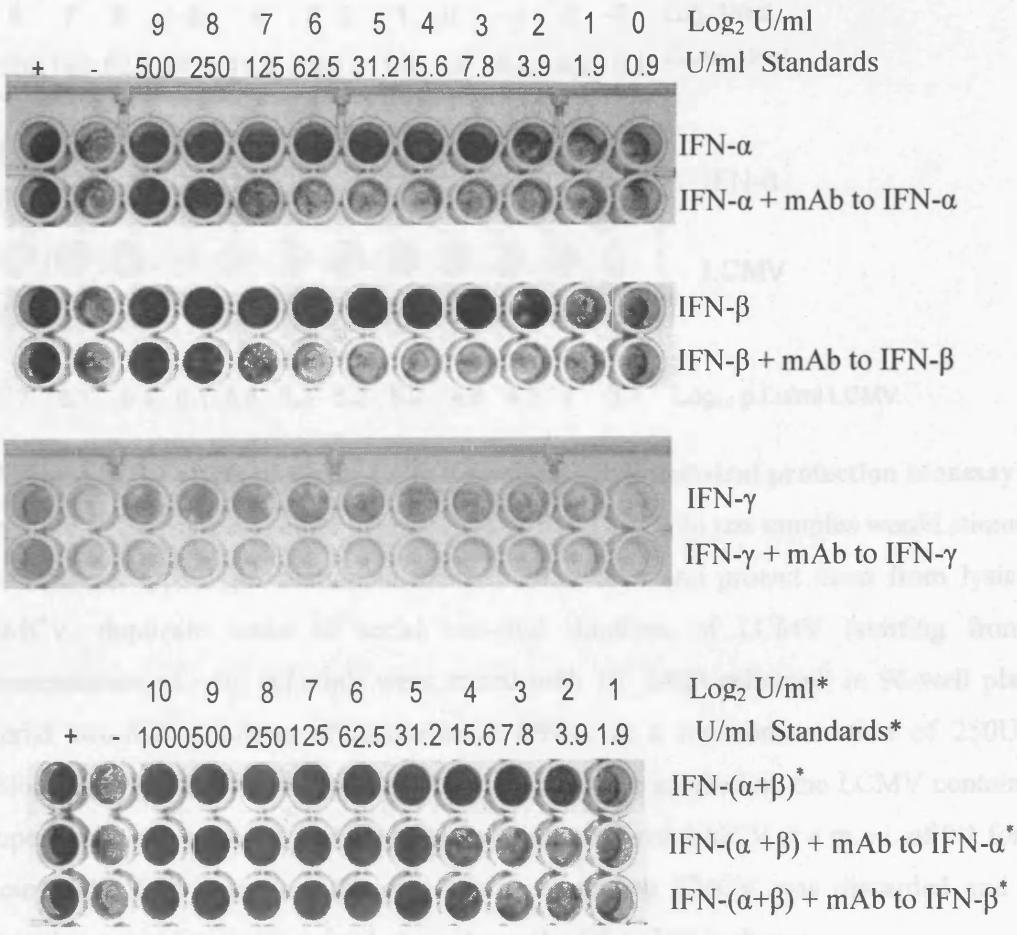


Figure 3.2 Standard curves of IFN-α, -β and -γ +/- neutralising antibodies in an EMCV antiviral bioassay

Serial two-fold dilutions of recombinant murine IFNs were prepared, starting from a top concentration of 500 U/ml (IFN-α , –β or –γ alone) or 1000 U/ml (IFN-(α + β) combined 1:1, marked with an *), and were added, with or without pre-incubation with the indicated neutralizing antibodies (mAb), and were added at a ratio of 2 neutralizing units per unit of IFN, to rows of L929 cells in 96 well microtitre plates (well 1= highest IFN concentration; well 10= lowest IFN concentration). After an overnight incubation the IFN containing supernatant was removed and the stimulated L929 cells were infected with EMCV at a m.o.i. of 0.1 at for 16-21 hours. The virus was then removed and the monolayer and stained. Wells marked '+' were positive control wells that were not infected with EMCV, and so possessed an intact monolayer while the wells marked '-' were negative control wells which did not receive IFN treatment prior to EMCV infection, resulting in complete lysis of the monolayer. The results shown are representative of findings made in at least 2 independent experiments.

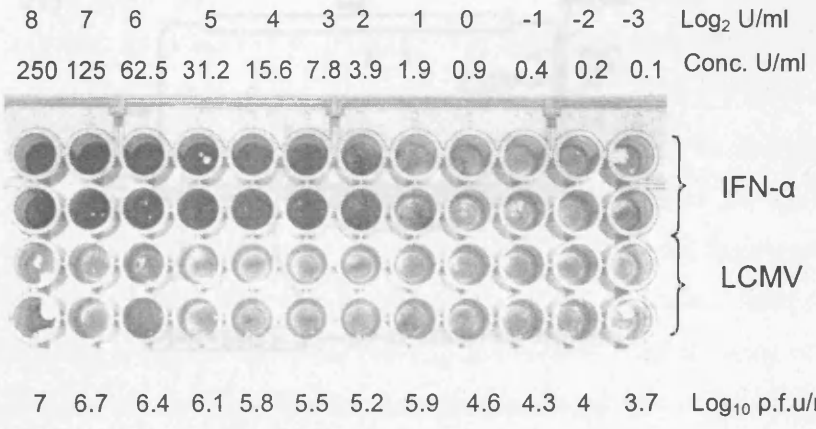


Figure 3.3 The effect of viable LCMV on the EMCV antiviral protection bioassay

In order to determine whether the presence of live LCMV in test samples would stimulate endogenous type 1 production in the test L929 cells and protect them from lysis by EMCV, duplicate wells of serial two-fold dilutions of LCMV (starting from a concentration of ~10^7 p.f.u/ml) were mixed with 10^5 L929 cells/well in 96-well plates. Serial two-fold dilutions of recombinant IFN-α, at a top concentration of 250U/ml ($8\log_2$U/ml) were set up in parallel. After an overnight incubation, the LCMV containing supernatant was removed and the cells were infected with EMCV at a m.o.i. of 0.1 for 16 hours. The following day, the supernatant containing EMCV was discarded and the monolayer was fixed and stained. A photograph of the plate is shown.

The titre of LCMV in the relevant wells are shown at the bottom of the plate, expressed as $\log_{10}$ p.f.u/ml virus. The concentration of IFN-α in the corresponding wells are shown above the figure, expressed as U/ml or converted to $\log_2$U/ml.

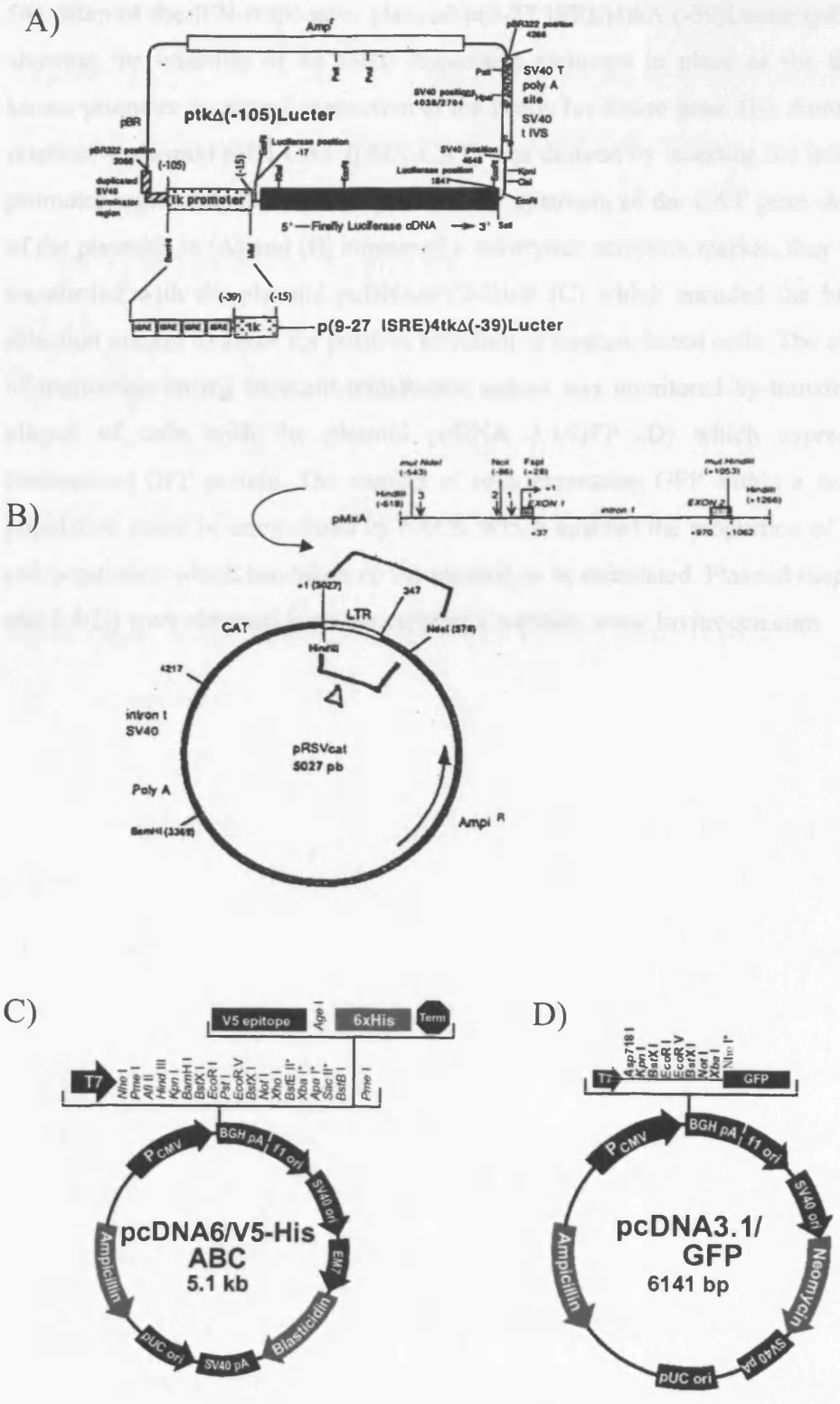


Figure 3.4 A-D Plasmids employed in the development of IFN-α/β reporter gene assays (figure legend overleaf)

(A). Map of the IFN-responsive plasmid p(9-27 ISRE)4tkΔ (-39)Lucter (pISRE-luc) showing the insertion of 4x ISRE responsive elements in place of the thymidine kinase promoter to control expression of the firefly luciferase gene. (B). Another IFN-responsive plasmid pHH-CAT (pMX-CAT) was derived by inserting the human Mx-promoter region into the plasmid pRSV-CAT upstream of the CAT gene. As neither of the plasmids in (A) and (B) contained a eukaryotic selection marker, they were co-transfected with the plasmid pcDNA6/V5-HisB (C) which encoded the blasticidin selection marker to allow for positive selection of co-transfected cells. The efficiency of tranfection during transient transfection assays was monitored by transfecting an aliquot of cells with the plasmid pcDNA 3.1/GFP (D) which expresses the luminescent GFP protein. The number of cells expressing GFP within a transfected population could be enumerated by FACS, which enabled the proportion of the total cell population which had taken up the plasmid to be calculated. Plasmid maps 3.4(C) and 3.4(D) were obtained from the supplier's website, www.Invitrogen.com

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L929 were also stimulated with media containing 10 μg/ml polyIC, 10 μg/ml R848, 2 nmol/ml CpG 1826 or 10 μg/ml LPS that had previously been incubated at 37°C for 24 hours to mimic the conditions of an *in-vitro* stimulation experiment, and the results indicate that the cells were not stimulated to produce endogenous IFN by any residual amounts of TLR ligand still present in the supernatant after 24 hours at 37°C (data not shown).

The results presented above indicate that the bioassay is a more sensitive assay than the IFN-α ELISA (detection limit of ~4 U/ml IFN-α versus ~100 U/ml (2.8 pg/ml) by the laboratory-based IFN-α ELISA). Nonetheless, the limit of detection of the bioassay may not be sensitive enough to detect very low levels of type 1 IFN in experimental samples. I therefore decided to develop a reporter gene-based assay capable of sensing very low levels of murine type 1 IFNs.

3.4 Development of reporter gene-based assays for sensitive detection of type 1 IFNs

For quantitation of type 1 IFN activity, reporter gene-based assays were set up where cells are transfected with a plasmid containing a reporter gene such as luciferase (Figure 3.4 A) or CAT (Figure 3.4 B) under the control of an IFN-responsive promoter and type 1 IFN activity is quantitated on the basis of the level of gene expression it stimulates in transfected cell. I worked with two reporter plasmids MX-CAT or ISRE-luc. The CAT reporter in the MX-CAT plasmid is linked to the promoter for the Mx gene, an anti-viral protein upregulated by type 1 IFNs while the luciferase gene is controlled by the 4 copies of the core 9-27 ISRE sequence of AGGAAATAGAAACTG which was engineered to remove the IFN-γ response

element (King and Goodbourn 1994). Stocks of these plasmids were generated as described in Section 2.4.5.

Reporter gene-based assays using these plasmids could have several advantages over the classical antiviral bioassay: firstly, they represent a more direct method of detecting type 1 IFN activity; secondly, they may be more specific as the reporter gene is only activated by the presence of type 1 IFN; and finally, they could be more sensitive as the signal can be amplified through the presence of multiple copies of the plasmid in a single cell.

3.4.1 Setting up a reporter assay system using transiently transfected cells

The most common method of utilizing the ISRE-luc plasmid to quantitate human type 1 IFN activity is by transiently transfecting the plasmid into human 293 cells and then stimulating the transiently transfected cells with experimental samples and known quantities of human type 1 IFN to generate a standard curve from which levels of type 1 IFN in the experimental samples can be determined (Andrejeva et al. 2004; Deonarain et al. 2000; Didcock et al. 1999a; b; Poole et al. 2002).

However, certain species of type 1 IFN do not cross recognize other species (Bridgman et al. 1988; Proietti et al. 1986). For example, human but not murine type 1 IFNs are recognized and promote signalling by the type 1 IFN receptor on the bovine kidney cell line MDBK (my unpublished observations). As such, in order to develop assays for quantitation of murine type 1 IFN activity, I decided to transiently transfect the MX-CAT or ISRE-luc plasmids into a murine cell line. The promoter sequence of the Mx gene was cloned from murine Mx gene (Lleonart et al. 1990), while the ISRE was derived from human sequences, however, pilot experiments using

transiently transfect cells showed that these would still be recognized by intracellular transcription factors in mouse cells (my unpublished observation).

The plasmids ISRE-luc or Mx-CAT were transfected into early passage murine BalbCl7 cells, an adherent fibroblast cell lines that had previously been shown to give the best transfection efficiencies of a panel of murine cell lines evaluated (Guyver 2001). In order to monitor the efficiency of transfection in each experiment, a well of BalbCl7 cells were transfected with the GFP plasmid pcDNA-GFP (Figure 3.4D) at similar DNA:lipid ratios as used for the reporter gene plasmids. I observed large assay to assay variation in the transfection efficiencies which typically ranged from 40% to 80% as shown in Figure 3.5. More importantly, the transfection process itself stimulated a moderately high level of reporter gene expression (which may potentially have been due to stimulation of endogenous type 1 production by BalbCl7 cells) and although the response to exogenously added recombinant IFN-α or IFN-β could be observed above this, the increases observed were in consistent and reproducible, quantitative IFN-α or IFN-β standard curves could not be generated (Figure 3.6). Another drawback was that the assay only worked when the cells were plated out into individual wells of 6-well plates and then transfected one at a time, making this strategy both costly and impractical for the analysis of large numbers of samples.

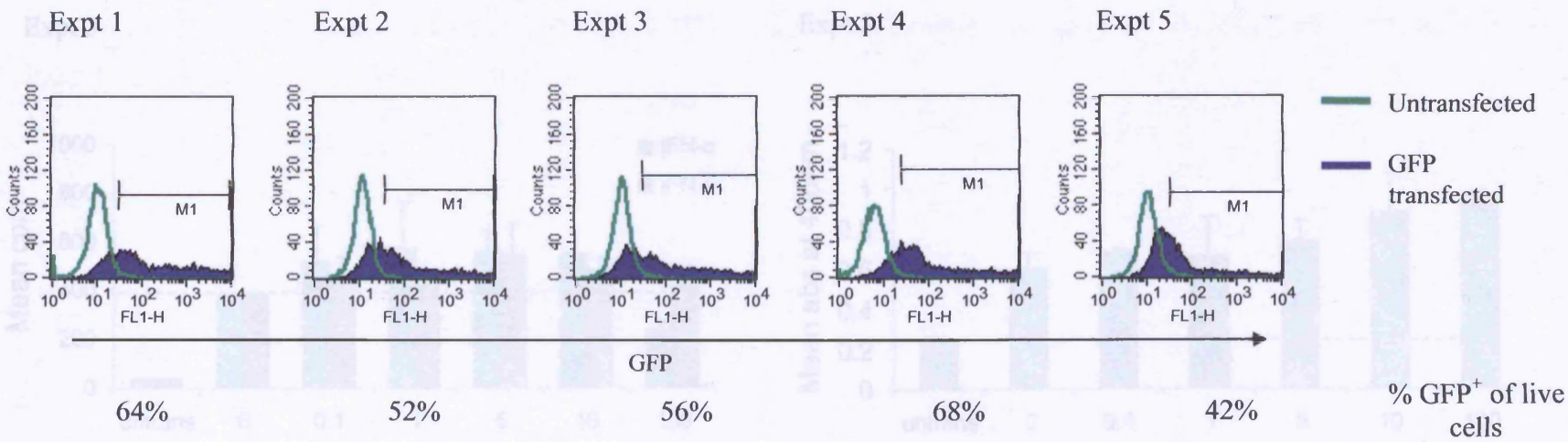

Figure 3.5 The efficiencies of transfection of BalbCl7 cells in different experiments

When BalbCl7 cells were transfected with a type 1 IFN-responsive plasmid in 6-well plates, a well of cells was transfected in parallel with the GFP-reporter plasmid to assess the efficiency of transfection in each experiment. The number of cells expressing GFP at 24 hours post transfection was determined by FACS analysis, comparing the fluorescence of the transfected cells (shaded histograms) to that of non-transfected cells (open histograms). The M1 marker was set relative to the uninfected cell population. The percentage of GFP-transfected cells falling within the M1 region is shown beneath each histogram.

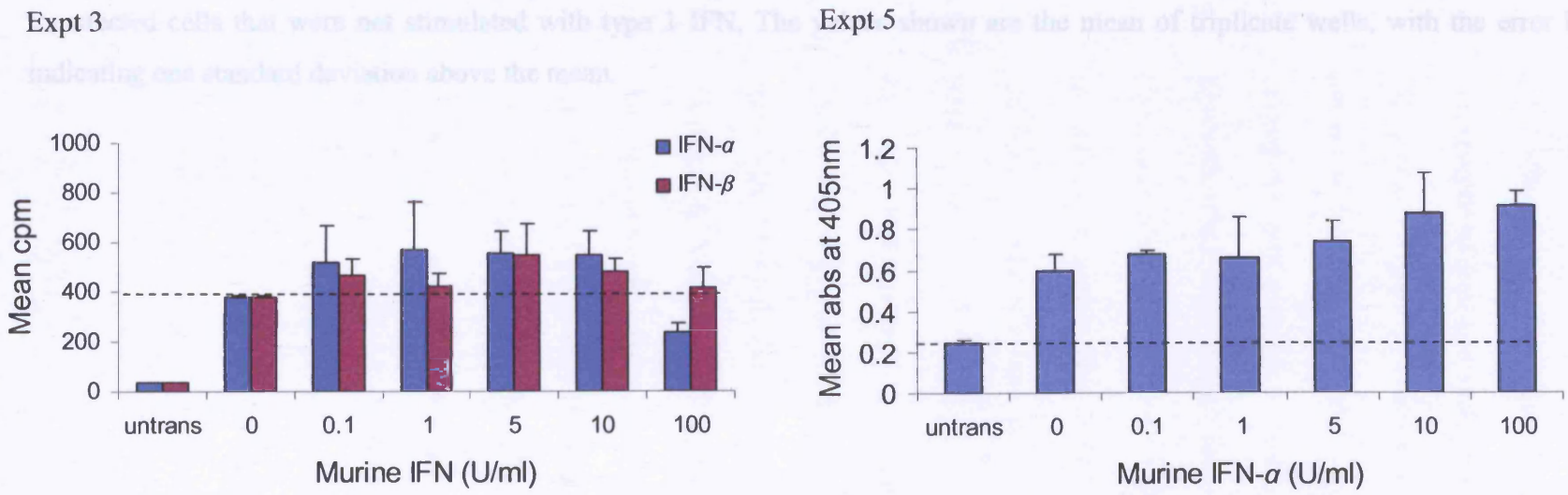

Figure 3.6 Responses of BalbCl7 transiently transfected with the pISRE-luc or pMX-CAT plasmids to recombinant IFN-α or IFN-β

BalbCl7 cells transiently transfected with the pISRE-luc (Expt 3) or pMX-CAT (Expt 5) plasmids in 6-well plates were stimulated the following day with serial dilutions of murine IFN-α or β. After an overnight incubation the cells were lysed and expression of the respective reporter genes assayed. Lysates from stimulated cells transfected with pISRE-luc (Expt 3) were quantitated using the Luciferase Assay system (results expressed as counts per minute, cpm), while a CAT ELISA was used to determine the reporter gene levels in stimulated pMX-CAT transfected transfected cells (Expt 5) (results expressed as absorbance values (abs) at 405nm). The results obtained have been plotted against the concentration of IFN used in the stimulation; 'untrans' represents the background counts or absorbance observed when untransfected cells were assayed and 0 indicates the level of reporter gene expression observed in

transfected cells that were not stimulated with type 1 IFN. The values shown are the mean of triplicate wells, with the error bars indicating one standard deviation above the mean.

3.4.2 Generation of cell lines stably transfected with reporter gene constructs and analysis of their responsiveness to type 1 IFNs

As quantitating type 1 IFN activity using transiently transfected BalbC17 cells did not prove a workable plan, I decided to generate BalbC17 cell lines stably transfected with the reporter gene plasmids to obtain more consistent results in a practical manner. While the ISRE-luc construct has only been used in transient transfection assays (Deonarain et al. 2000; Didcock et al. 1999a; Poole et al. 2002; Young et al. 2001), several other groups have developed reporter gene assays utilizing stably transfected cell lines to measure human and bovine type 1 IFN (Baigent et al. 2002; Meager 2002).

As neither of the reporter constructs used here contains a eukaryotic cell selection marker, they were co-transfected with a plasmid encoding a blasticidin resistance gene (Figure 3.4C) so stably that transfected cells could be selected on the basis of resistance to blasticidin by culture in blasticidin supplemented media. Several blasticidin-resistent colonies were isolated from each transfection, and these were initially pooled together and assayed for quantitative responses to recombinant and natural IFN-α and -β. The cells were seeded into 6-well plates, treated overnight with a range of IFN-α and -β concentrations and then they were lysed and reporter gene expression assayed. Luciferase activity was detected by luminometry while an anti-CAT ELISA was used to detect CAT expression in BalbC17 MX-CAT cell lysates. Both cell lines gave dose dependent responses to IFN-α and –β, with the limit of detection at approximately 10U (data not shown).

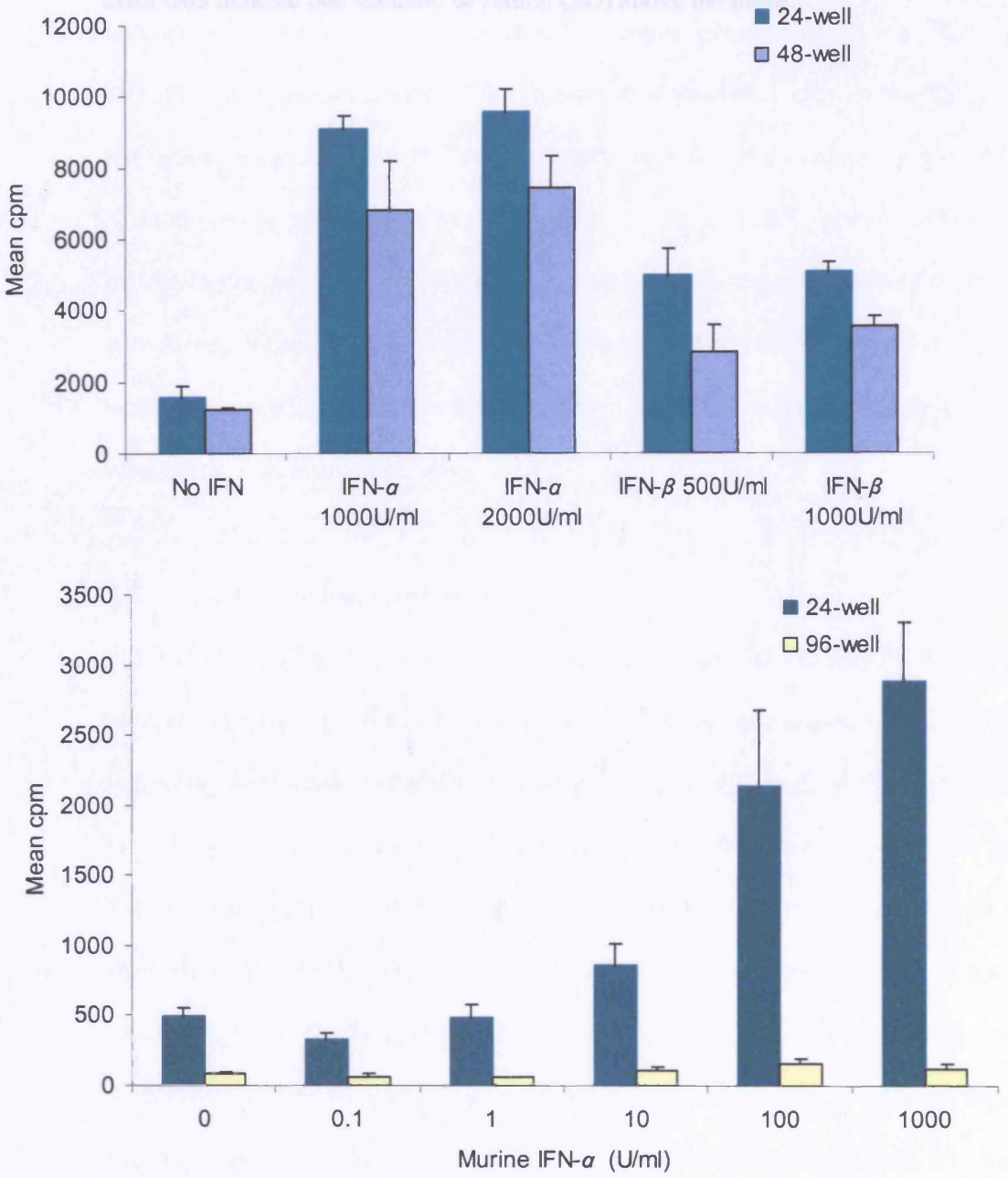

Figure 3.7 Response of BalbCl7 ISRE-luc cells to type 1 IFN as detected in assays carried out in plates with wells of differing sizes

BalbCl7 ISRE-luc cells were seeded into 24-, 48- and 96-well plates at cell concentrations of $4x10^4$, $2x10^4$ and $8x10^3$ cells per well respectively. The following day, the cells were stimulated with recombinant murine IFN-α or IFN-β protein. After

a 24 hour incubation, cells were lysed and luciferase production was measured by luminescence assay. The results are shown as mean cpm from triplicate wells; the error bars indicate one standard deviation (SD) above the mean.

The initial assay was performed in 6-well plates and I subsequently adapted it to 24-well plates and them attempted to scale down the assay format to allow samples to be tested in 48- or 96-well plates. Cell concentrations were scaled down from the 6-well format by the calculating ratio of the number of cells/surface area. Accordingly, 48-well plates were seeded with $2x10^4$ cells/well in 500µl media while 96-well plates (Corning) were seeded with $8x10^3$ cells/well in 100µl media. The 48-well format produced similar quantitative responses to IFN-α or –β to those observed in the 24-well format (Figure 3.7). Attempts to scale the assay down further into 96-well plates were not successful, as the levels of luciferase induction obtained in this format were minimal and were not quantitative.

3.4.3 Selection of single colonies

The pooled blasticidin resistant cells initially gave reproducible responses to IFNs, however with time, the BalbC17 cells appeared to become less responsive to IFN, suggesting that a large proportion of the heterogenous cell population had been taken over by non-responders. We recovered aliquots of cells frozen at ~4 weeks post blasticidin selection and cloned these cells by limiting dilution. Single cell colonies were identified, 30 for BalbC17 transfected with ISRE-luciferase (BalbC17 ISRE-luciferase) and 64 for BalbC17 transfected with MX-CAT (BalbC17 MX-CAT). These were expanded into 24- well plates and tested for their response to 500U/ml of IFN-α, Figure 3.8 shows an example of the results from the screening of BalbC17 ISRE-luciferase cells and Figure 3.9 an example of results from the screening of BalbC17 MX-CAT clones. We found that most of the blasticidin resistant cells only contained the blasticidin resistence gene without a reporter plasmid. However, a number of doubly transfected clones were detected which gave good responses to type 1 IFN

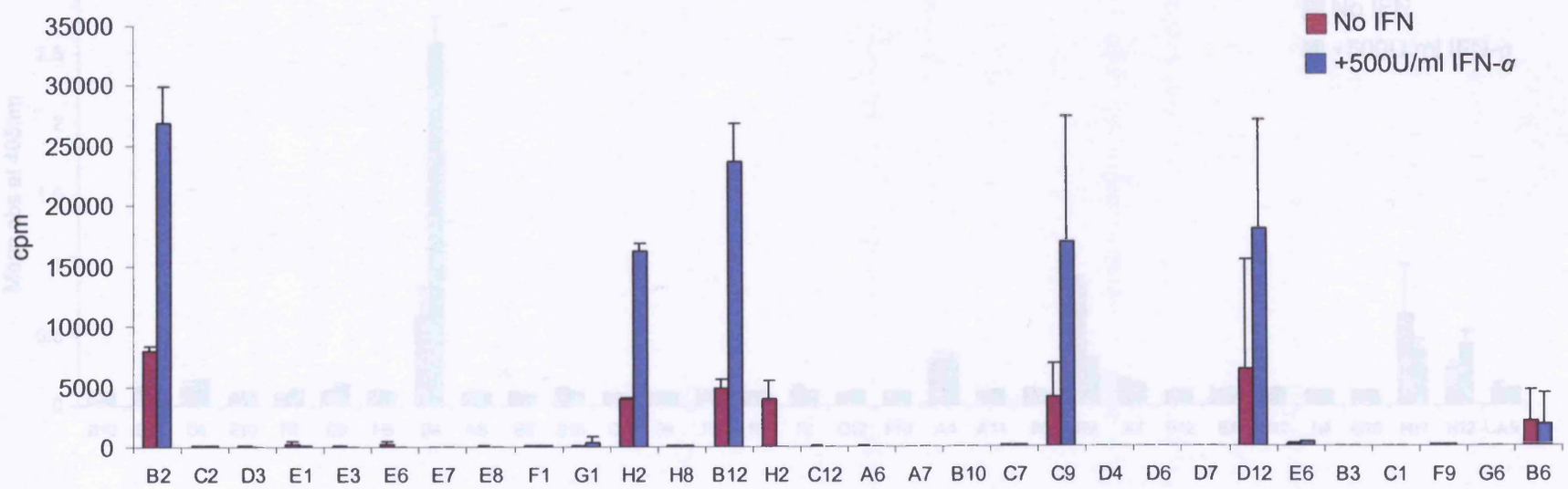

Figure 3.8 Screening clones of BalbCl7 cells transfected with the ISRE-luc plasmid (BalbCl7-ISRE-luc) for responsiveness to IFN-α

Single-cell colonies generated by limiting dilution growth of BalbCl7 cells transfected with an ISRE-luc plasmid were expanded into replicate wells in 48-well plates. When they were about 40% confluent, cells in duplicate wells were stimulated with 500U/ml of recombinant IFN-α for 24 hours; other wells of cells were left untreated. Luciferase expression in IFN-treated and untreated wells was analysed by luminescence assay and the results are expressed as counts per minute (cpm). Clones were tested in duplicate and the error bars indicate one SD above the mean.

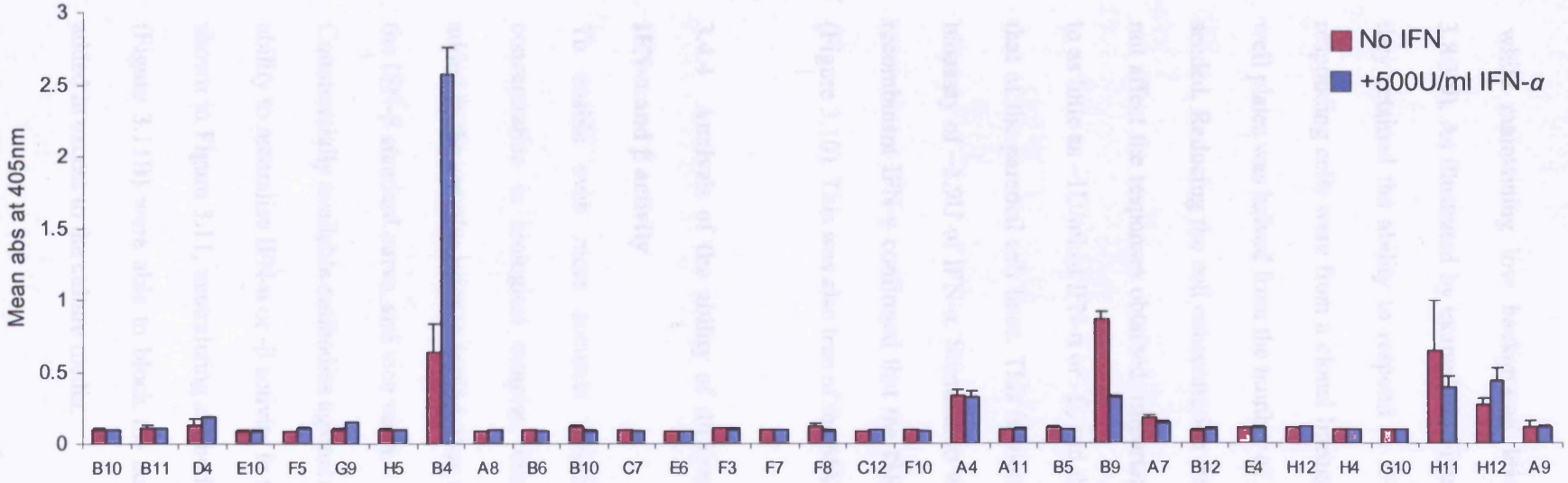

Figure 3.9 Screening clones of BalbCl7 cells transfected with plasmid MX-CAT (BalbCl7-MX-CAT) for responsiveness to IFN-α

Single-cell colonies generated by limiting dilution growth of BalbCl7 cells transfected with HH-CAT plasmid were expanded into replicate wells in 48-well plates. When they were about 40% confluent, cells in duplicate wells were stimulated with 500U/ml of recombinant IFN-α for 24 hours; other wells of cells were left untreated. The level of CAT expression in IFN-treated and untreated wells was analysed by CAT ELISA and the results are expressed as the absorbance (abs) at 405nm. Clones were tested in duplicate and the error bars indicate one SD above the mean.

while maintaining low background levels of reporter gene expression (Figure 3.8/3.9). As illustrated by example in Figure 3.10, when these clones were expanded they retained the ability to respond to IFN-α in a dose dependent fashion. As the responding cells were from a clonal lineage, the total number of cells seeded into 48-well plates was halved from the number of uncloned parental cells that was previously seeded. Reducing the cell concentration from $2x10^4$ cells/well to $1x10^4$ cells/well did not affect the responses obtained. Importantly, the clones gave a detectable response to as little as ~1U/ml of IFN-α or –β, and their responses were more reproducible than that of the parental cell lines. This is more sensitive than the visual endpoint of the bioassay of ~3.9U of IFN-α. Stimulation with recombinant serial 10-fold dilutions of recombinant IFN-γ confirmed that the ISRE-luc promoter did not respond to IFN-γ (Figure 3.10). This was also true of the MX-CAT promoter (data not shown).

3.4.4 Analysis of the ability of different anti-IFN antibodies to block murine IFN-α and β activity

To enable even more accurate determination of the IFN composition and concentration in biological samples, neutralising anti-IFN-α antibodies could be added to the samples prior to testing, thus allowing IFN-β levels to be quantitated off the IFN-β standard curve, and vice versa.

Commercially available antibodies against murine IFNs were tested to determine their ability to neutralise IFN-α or -β activity in the reporter gene assays (Figure 3.11B). As shown in Figure 3.11, neutralizing antibodies against IFN-α (Figure 3.11A) or IFN-β (Figure 3.11B) were able to block the activity of their respective IFN types when added in excess to the culture media.

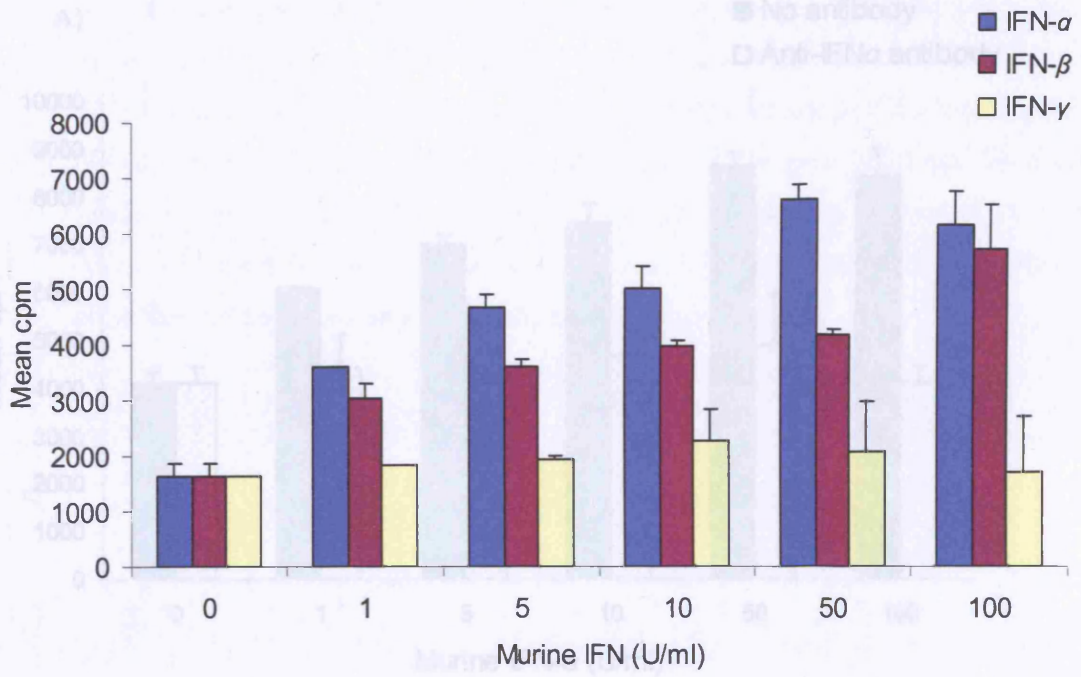

Figure 3.10 Response of BalbCl7-ISRE-luc clone B2 to recombinant murine IFN-α,-β and -γ

The cells were plated into 48-well plates at a concentration of $1x10^4$cells/well. The following day, cells were stimulated with serial dilutions of a) recombinant mouse IFN-α or b) recombinant murine IFN-β in 200μl of growth medium. After a 24 hour incubation, the cells were lysed and the amount of luciferase reporter expression was determined by luminescence assay. The results are expressed as mean counts per minute (cpm). The data shown are mean values from duplicate wells; the error bars indicate one standard deviation (SD) above the mean.

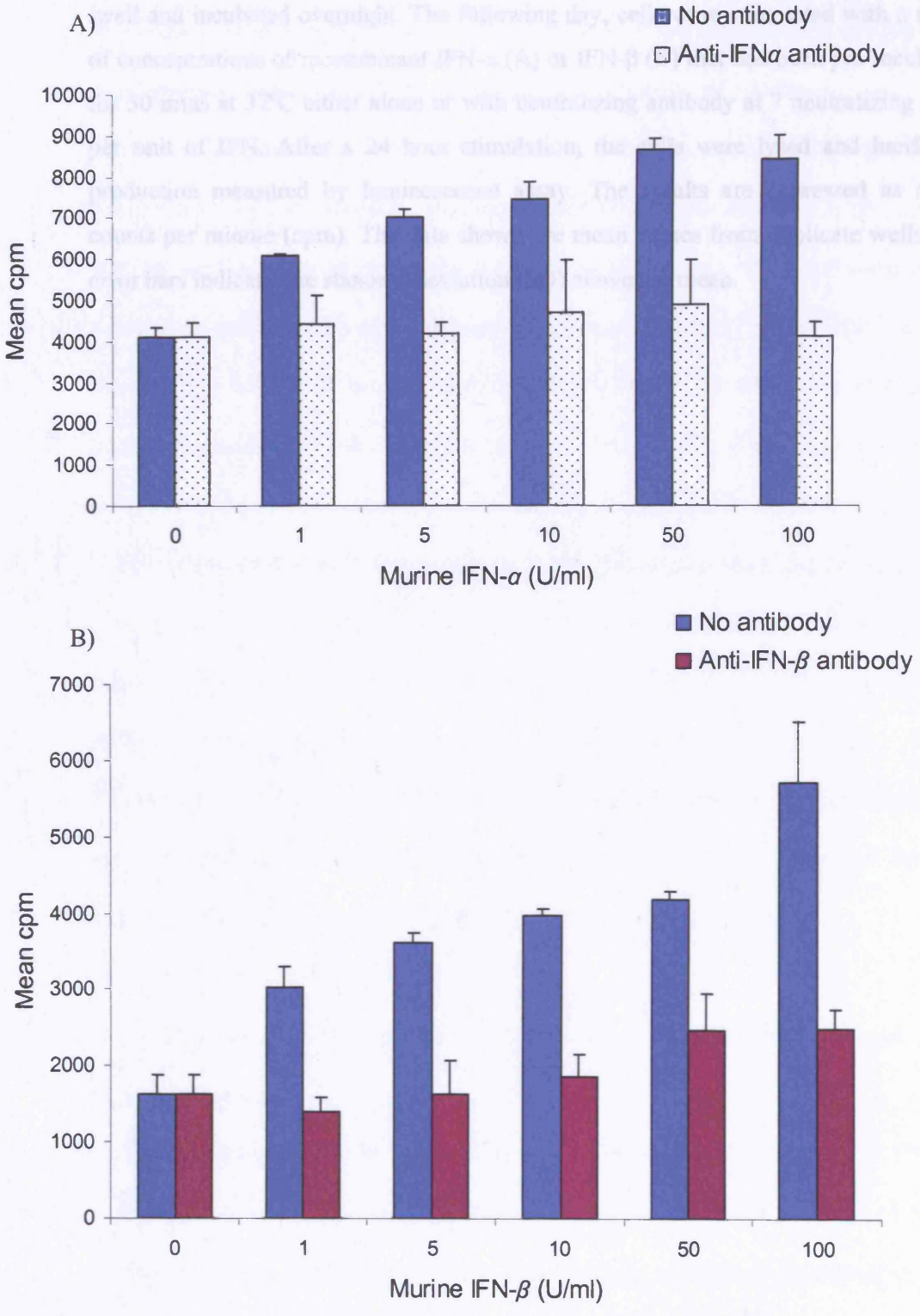

Figure 3.11 A) and B) Neutralizing antibodies abrogate the response of BalbCl7 ISRE-luciferase cells to type 1 IFNs (legend on following page)

BalbC17 ISRE-luciferase clone B2 cells were seeded into 48-well plates at $1x10^4$ cells /well and incubated overnight. The following day, cells were stimulated with a range of concentrations of recombinant IFN-α (A) or IFN-β (B) that had been pre-incubated for 30 mins at 37°C either alone or with neutralizing antibody at 7 neutralizing units per unit of IFN. After a 24 hour stimulation, the cells were lysed and luciferase production measured by luminescence assay. The results are expressed as mean counts per minute (cpm). The data shown are mean values from duplicate wells; the error bars indicate one standard deviation (SD) above the mean.

3.4.5 Effects of passaging on the sensitivity of the reporter cells

It was discovered over time that constant passaging of the reporter cells in blasticidin supplemented media resulted in higher backgrounds of luciferase and CAT expression, leading to the cells losing their sensitivity to type 1 IFN. Figure 3.12 compares the IFN–α standard curve from BalbCl7 ISRE-luciferase at a late passage with an identical experiment conducted using early passage cells of the same clone. While early passage cells were responsive to at least 2.5U of IFN-α or IFN-β, cells at later passage could only respond to >50U of IFN-α or IFN-β, due to the background counts increasing by 3-fold. Other people have observed this with other cell lines (Dr Steve Goodbourn, St George's Medical School, London, personal communication); it is believed to be due to endogenous type 1 IFN production which occurs despite the measures taken to ensure the system is free from potential type 1 IFN inducing stimuli, which included culturing the cells with polymyxin B in low-endotoxin, BVDV-free serum and keeping the cells at log-phase, sub-confluent growth.

The loss of sensitivity in these stably transfected cells as they were passaged rendered the reporter assays unreliable. It was therefore decided that they were not suitable for measuring the levels of secreted type 1 IFN in experimental samples.

3.5 Real-time PCR detection of different type 1 IFN subtypes and IFN-stimulated genes

In parallel with efforts to develop assays to measure secreted type 1 IFN in supernatant or serum, optimization of real-time quantitative PCR reactions to detect mRNA of IFN-β and specific IFN-α subtypes was conducted. Quantitative PCR reactions were designed to allow measurement of levels of expression of IFN-β and

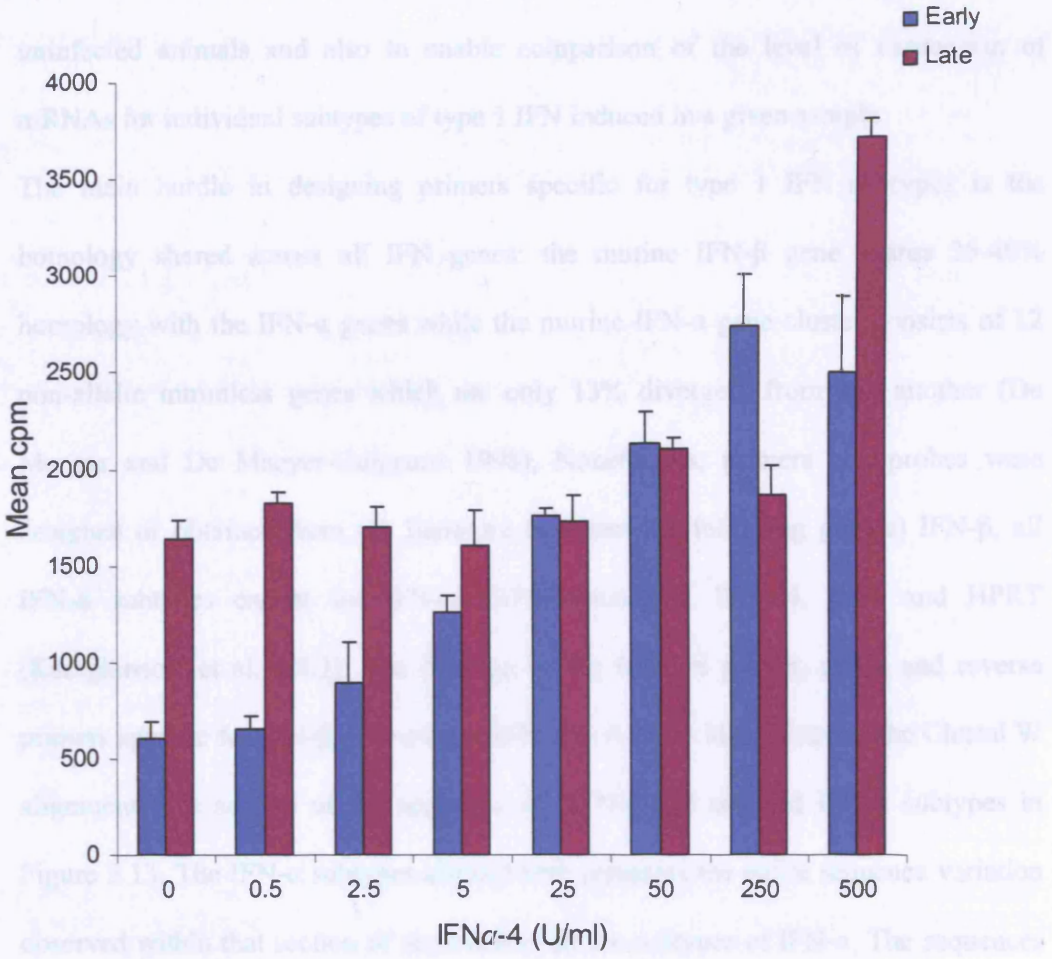

Figure 3.12 Comparison of the response of early and late passage BalbC17 ISRE-luciferase clone B2 cells to IFN-α4

Early passage BalbC17 clone B2 cells (~5 passages after clone was selected and expanded) and cells that had been passaged an additional 5 times were seeded at $1x10^4$ cells/well into a 48-well plate. The following day, the cells were stimulated with serial dilutions of murine IFN-α4 in 200μl of media. After a 24 hour incubation period, cells were washed, lysed and the amount of luciferase was determined by luminescence assay. The results shown are the mean counts per minute (cpm) from triplicate wells; the error bars indicate one standard deviation (SD) above the mean.

specific IFN-α subtype trancripts in *in vitro* cultured cells or tissues from infected vs. uninfected animals and also to enable comparison of the level of expression of mRNAs for individual subtypes of type 1 IFN induced in a given sample.

The main hurdle in designing primers specific for type 1 IFN subtypes is the homology shared across all IFN genes: the murine IFN-β gene shares 25-40% homology with the IFN-α genes while the murine IFN-α gene cluster consists of 12 non-allelic intronless genes which are only 13% divergent from one another (De Maeyer and De Maeyer-Guignard 1998), Nonetheless, primers and probes were designed or obtained from the literature to detect the following gene(s) IFN-β, all IFN-α subtypes except for IFN-α4 (IFN-α(non-α4)), IFN-α4, Mx1 and HPRT (Karaghiosoff et al. 2003). The location of the forward primer, probe and reverse primers specific for IFN-β, IFN-α4 and IFN-α(non-α4) is highlighted in the Clustal W alignment of a section of the sequence of IFN-β and selected IFN-α subtypes in Figure 3.13. The IFN-α subtypes aligned here represent the entire sequence variation observed within that section of sequence in all the subtypes of IFN-α. The sequences were confirmed by BLAST to be specific for their respective genes. The IFN-β probe was labelled with the fluorescent dyes FAM on the 5' end and TAMRA on the 3' end while the probes for IFN-α4 and IFN-α(non-α4) were labelled with FAM on the 5' end and TAMRA plus a minor groove binder (MGB) at the 3' end to provide increased sequence specificity (Kutyavin et al. 2000).

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gi|6680368|ref|NM_008335.1|(a6)      AGGCTGTCTGATGCAGCAGGTAGAGATACAGGCACTTCCCCTGACCCAGG 477
gi|220441|dbj|D00460.1|MUSIFNA8      AGGCTGTCTGATGCAGCAGGTAGAGATACAGGCACTTCCCCTGACCCAGG 963
gi|51546|emb|X01971.1|MMIFNA5        AGCCTGTGTGATGCAACAGGTCGGGGTGCAGGAATCTCCCCTGACCCAGG 109
gi|6754293|ref|NM_010504.1|(a4)      AGCCTGTGTGATGCA--------------GGAACCTCCTCTGACCCAGG 397
gi|6754291|ref|NM_010503.1|(a2)      AACCTGTCTGATGCAGCAGGTGGGGGTGCAGGAACCTCCTCTGACCCAGG 409
gi|6754303|ref|NM_010510.1|(beta)    AGACAGTACTAGAGGAAAAG--------CAAGAGGAAAGATTGACGTGGG 408
                                     *  * **   *                 *          ****   **

gi|6680368|ref|NM_008335.1|(a6)      AAGACTCC----CTGCTGGCTG--TGAGGACATACTTCCACAGGATCACT 521
gi|220441|dbj|D00460.1|MUSIFNA8      AAGACTCC----CTGCTGGCTG--TGAGGACATACTTCCACAGGATCACT 100
gi|51546|emb|X01971.1|MMIFNA5        AAGACTCC----CTGCTGGCTG--TGAGGAAATACTTCCACAGGATCACT 113
gi|6754293|ref|NM_010504.1|(a4)      AAGACTCC----CTGCTGGCTG--TGAGGACATACTTCCACAGGATCACT 441
gi|6754291|ref|NM_010503.1|(a2)      AAGACGCC----CTGCTGGCTG--TGAGGAAATATTTCCACAGGATCACT 453
gi|6754303|ref|NM_010510.1|(beta)    AGATGTCCTCAACTGCTCTCCACTTGAAGAGCTATTACTGGAGGGTGCAA 458
                                     *     **    *****  *    *** **  ** * *   *** *

gi|6680368|ref|NM_008335.1|(a6)      GTGTTCCTGAGAGAGAAGAAACACAGCCCCTGTGCCTGGGAGGTGGTCAG 571
gi|220441|dbj|D00460.1|MUSIFNA8      GTGTTCCTGAGAGAGAAGAAACACAGCCCCTGTGCCTGGGAGGTGGTCAG 105
gi|51546|emb|X01971.1|MMIFNA5        GTGTACCTGAGAGAGAAGAAACACAGCCCCTGTGCCTGGGAGGTGGTCAG 118
gi|6754293|ref|NM_010504.1|(a4)      GTGTACCTGAGAAAGAAGAAACACAGCCTCTGTGCCTGGGAGGTGATCAG 491
gi|6754291|ref|NM_010503.1|(a2)      GTGTACCTGAGAGAGAAGAAACACAGCCCCTGTGCCTGGGAGGTGGTCAG 503
gi|6754303|ref|NM_010510.1|(beta)    AGGTACCTTAAACTCATGAAGTACAACAGCTACGCCTGGATGGTGGTCCG 508
                                       ** *** * *   * ***  *** *  **  ******  **** ** *

gi|6680368|ref|NM_008335.1|(a6)      AGCAGAAGTCTGGAG-AGCCCTGTCTTCCTCAGCCAAGTTGCTGGCAAGA 620
gi|220441|dbj|D00460.1|MUSIFNA8      AGCAGAAGTCTGGAG-AGCCCTGTCTTCCTCAGCCAAGTTGCTGGCAAGA 110
gi|51546|emb|X01971.1|MMIFNA5        AGCAGAAGTCTGGAG-AGCCCTGTCTTCCTCAGTTAACTTGCTGGCAAGA 123
gi|6754293|ref|NM_010504.1|(a4)      AGCAGAAGTCTGGAG-AGCCCTCTCTTCCTCAACCAACTTGCTGGCAAGA 540
gi|6754291|ref|NM_010503.1|(a2)      AGCAGAAGTCTGGAG-AGCCCTGTCTTCCTCAGTCAACTTGCTGCCAAGA 552
gi|6754303|ref|NM_010510.1|(beta)    AGCAGAGATCTTCAGGAACTTTCTCATCATTCGAAGACTTACCAGAAACT 558
                                     ******  ***  ** * *  * ** ** *      * ** *    **

gi|6680368|ref|NM_008335.1|(a6)      CTGAA---TGAGGACGAGTGAGTCCTGAGACCAAGTGTGGAGAG-ACCTC 666
gi|220441|dbj|D00460.1|MUSIFNA8      CTGAA---TGAGGACGAGTGAGTCCTGAGACCAAGTGTGGAGAG-ACCTC 115
gi|51546|emb|X01971.1|MMIFNA5        TTGAG---CAAGGAGGAGTGACTCCTGAGACAAAGTGTGGAGAGGACTTT 128
gi|6754293|ref|NM_010504.1|(a4)      CTGAG---TGAGGAGAAG-GAGTGA------------------------- 561
gi|6754291|ref|NM_010503.1|(a2)      CTGAG---TGAAGAGAAG-GAGTGA------------------------- 573
gi|6754303|ref|NM_010510.1|(beta)    TCCAAAACTGAAGACCTGTCAGTTGATGCCTCAGAATGAGTGGTGGTTGC 608
                                        *      * **   *  * *

gi|6680368|ref|NM_008335.1|(a6)      CCTGGACTAGAACACTGCACCTGCACTTTATAAG--CTCCCTTTACAACT 714
gi|220441|dbj|D00460.1|MUSIFNA8      CCTGGACTAGAACACTGCACCTGCACTTTATAAG--CTCCCTTTACAACT 120
gi|51546|emb|X01971.1|MMIFNA5        TCTGAACCAGAACACTGCATCT-CACTTTATAAGATCTCCTTTAAAAACT 133
gi|6754293|ref|NM_010504.1|(a4)      ---------------------------------------------------
gi|6754291|ref|NM_010503.1|(a2)      ---------------------------------------------------
gi|6754303|ref|NM_010510.1|(beta)    AGGCAACCTTTAAGCATCAGAGGCGGACTCTGGGA-CTGGTAGTGAATCT 657


gi|6680368|ref|NM_008335.1|(a6)      CTCATTACCTTCA-GTGTGAACACAATCAACCTGCCAAGACGCTGCAGCA 763
gi|220441|dbj|D00460.1|MUSIFNA8      CTCATTACCTTCA-GTGTGAACACAATCAACCTGCCAAGACGCTGCAGCA 124
gi|51546|emb|X01971.1|MMIFNA5        CTCATAACTTTCACATATGAGTACAAACAACCTGCACAGATGTTTCAGCA 138
gi|6754293|ref|NM_010504.1|(a4)      ---------------------------------------------------
gi|6754291|ref|NM_010503.1|(a2)      ---------------------------------------------------
gi|6754303|ref|NM_010510.1|(beta)    ACTGCATTTGAAAGGTCAAAGGAAAACAGAGTTTTTATTAATTTATAAT- 706
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Figure 3.13 ClustalW alignment of cDNA or genomic sequences of selected IFN-α subtyp

Figure 3.13 ClustalW alignment of cDNA or genomic sequences of selected IFN-α subtypes and IFN-β

Multiple sequence alignment using ClustalW was conducted to identify conserved and heterogenous regions within the coding sequences of the genes for IFN-β and selected IFN-α subtypes; only the relevant portion of the alignment of the entire coding sequence is shown. The column to the left of the alignment identifies the gene through its Genbank designation, with the subtype name in brackets at the end of the desgination. The IFN-α subtypes aligned here represent all the sequence variation within the region shown. The nucleotide position of each of the aligned sequences is revealed in the column on the right of the alignment. Asterisks (*) beneath the alignment indicate the position is occupied by nucleotides that are conserved in all the sequences compared, while a dash (-) within the aligned sequence highlights a sequence deletion at that position. This information was applied to design primers and probes that were specific for the IFN-α(non-α4) subtypes only. The primer and probe sequence recognised by the IFN-α(non-α4) primer set is highlighted in red (coloured font = primer sequences, underlined coloured font = probe sequence), while the sequence recognised by the IFN-α4 probe/primers is coloured green and the region recognised by the IFN-β probe/primer set blue.

Initial optimization experiments determined the optimal primer concentration for each set. Figure 3.14 is an example of the optimization of the primer dilution for the IFN-α(non-α4) Taqman PCR; the value of slope of the line is a measure of the efficiency of the PCR reaction. The slopes for low dilutions of the primer set should be similar with a change in the slope indicating that the primer concentration limited the efficiency of the PCR reaction. Here, the minimum appropriate working dilution proved to be at 1/10 dilution or lower of the stock concentration. Ultimately, we decided to dilute the primers 1/5 of the stock concentration of 100pmol/μl, giving a final concentration of 20 pmol per reaction.

All type 1 IFN genes do not contain introns so primers that spanned exon-exon boundries could not be designed as a means to exclude genomic contamination. It therefore became imperative that all genomic DNA was removed from the samples, and this was achieved by an on-column DNAse digestion step during RNA isolation. In some samples, a second DNAse digestion step was performed on the aliquot of input RNA just before RT-PCR was conducted.

To check that IFN-β and IFN-α4 primer sets do not recognize non-α4 subtypes of IFN-α, they were tested against plasmids encoding the IFN-α2, -α5, -α6/8 or -α14 subtype (provided by Dr. Graeme Frith, Edward Jenner Institute, Compton, UK) in real-time PCR assay. As shown in Figure 3.15, positive responses against all the plasmids were obtained from the IFN-α(non-α4) primer set. The IFN-α4 primers did not recognize any of the non-α4 plasmids, and negative responses were also observed with the IFN-β primer set (data not shown). The primers for IFN-α4 and IFN-β were obtained from a published source, and the primer and probe sequences for the respective genes were confirmed by BLAST search to be specific for only the IFN

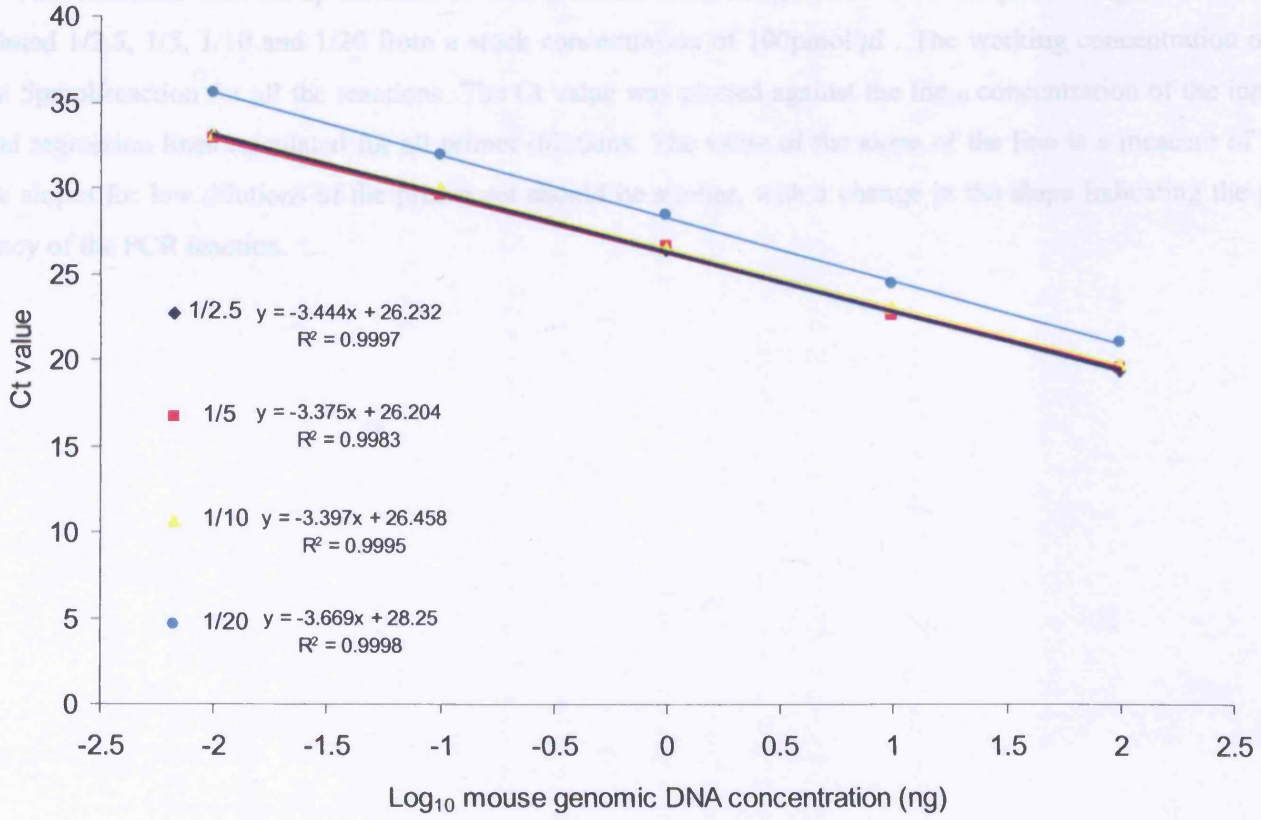

Figure 3.14 Optimization of primer dilutions for quantitative PCR

Working dilutions of primer sets for quantitative PCR were optimized to determine the lowest primer concentration which would not limit the efficiency of the PCR reaction. As a representative example, results of the experiment carried out for the optimization of the IFN-α(non-α4)

primers is shown. PCR reactions were set up on serial 10-fold dilutions of mouse genomic DNA template using forward and reverse primers of IFN-α(non-α4) diluted 1/2.5, 1/5, 1/10 and 1/20 from a stock concentration of 100pmol/μl . The working concentration of the IFN-α(non-α4) probe was fixed at 5pmol/reaction for all the reactions. The Ct value was plotted against the $\log_{10}$ concentration of the input template for each primer dilution and regression lines calculated for all primer dilutions. The value of the slope of the line is a measure of the efficiency of the PCR reaction. The slopes for low dilutions of the primer set should be similar, with a change in the slope indicating the primer concentration limited the efficiency of the PCR reaction.

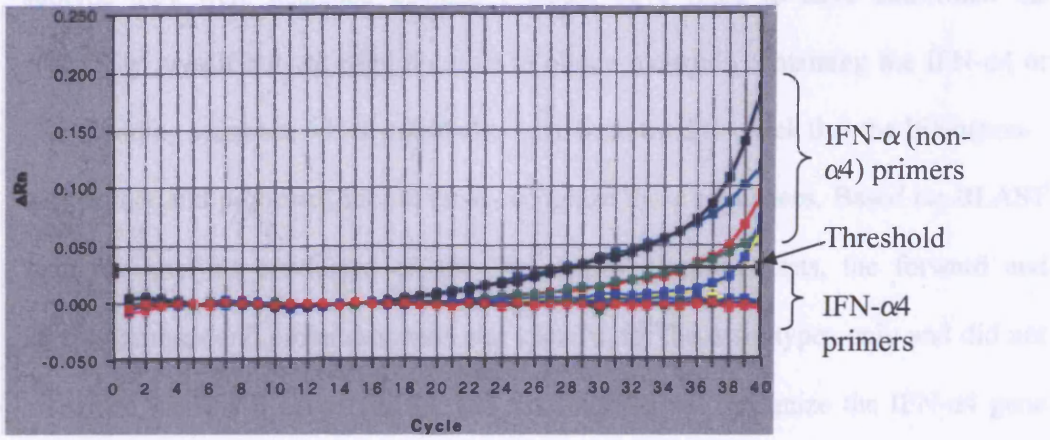

Figure 3.15 Amplification plot of IFN-α4 and IFN-α(non-α4) real-time PCR assays

To confirm the specificity of the IFN-α4 and the IFN-α(non-α4) quantitative PCR reactions, IFN-α4 or IFN-α(non-α4) primer sets were tested against 4 distinct plasmids encoding the IFN-α2, -α5, -α6/8 or -α14 subtype. Recognition of a subtype by the primer set results in the increase of the reaction's ΔRn above the set threshold during successive PCR cycles. The threshold was set according to criteria as explained in the Methods section 2.x. The threshold indicated is the threshold of the IFN-α(non-α4) reaction, which was set at 0.03, with the fluorescence detected during cycles 3-15 as the average baseline fluorescence. The threshold for the IFN-α4 reaction was set at 0.8, with the average fluorescence emissions during cycles 3-22 as the baseline. As illustrated above, the PCR reactions set up with the IFN-α(non-α4) primers crossed the threshold at cycle ~28 and at ~35. Using the threshold and baselines for the IFN-α4 reaction, the ΔRn values for all reactions using the IFN-α4 primer set remained negative.

subtype they were designed against. I would have liked to have confirmed the specificity myself but we were not able to obtain plasmids containing the IFN-α4 or IFN-β coding sequence which could also have been used to check that the IFNα(non-α4) primer and probe set did not cross-recognize those sequences. Based on BLAST sequence analysis conducted on the IFN-α(non-α4) primer sets, the forward and reverse primers and probe sequence was specific for the α subtypes only and did not recognize the IFN-β gene. The BLAST analysis did not recognize the IFN-α4 gene sequence as it contained a single nucleotide substitution in the forward and reverse primer binding regions (Figure 3.13). However, the PCR conditions may not have been stringent enough to prevent cross-recognition of the IFN-α4 subtype, so it remains a possibility that this primer set also recognizes the IFN-α4 subtype.

I next investigated the feasibility of directly comparing the levels of each IFN type measured to those of the other IFN types/subtypes. For a valid comparison of the levels of different IFN subtypes, the rate or efficiency of the PCR amplification process would need to be similar in all the primer sets. In order to determine the efficiencies of each primer set, they were tested against wild-type mouse genomic DNA which would contain an equal number of copies of the IFN-β and each of the IFN-α intronless gene. Taqman real-time PCR assays were conducted for each of the primer sets in PCR mastermix with 10-fold dilutions of genomic DNA from wild-type C57BL/6 mice (provided by Miranda Ashton, Edward Jenner Institute, Compton) as template. The Ct values were determined and plotted against the $\log_{10}$ concentration of input genomic DNA as template to generate standard curves for each primer set. As can be seen in Figure 3.16, the IFN-β and IFN-α (non-α4) primer set have similar slope values indicating the PCR reactions are of similar efficiencies. The curves are parallel and not superimposed because the IFN-β primer set is specific for the IFN-β

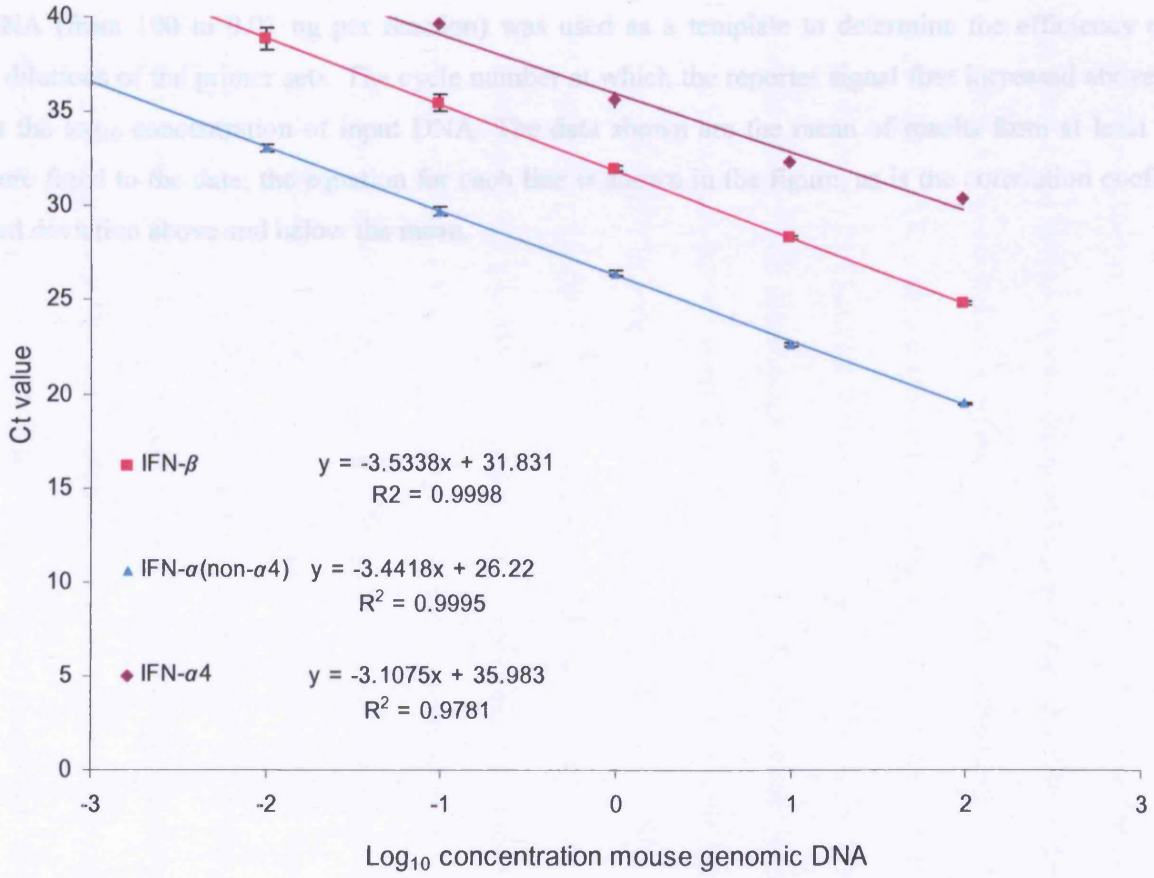

Figure 3.16 Standard curves for real time PCR analysis of IFN-β, IFN-α(non-α4) and IFN-α4 subtypes performed with 10-fold serial dilutions of mouse genomic DNA (legend on following page)

Mouse genomic DNA (from 100 to 0.01 ng per reaction) was used as a template to determine the efficiency of each PCR reaction using optimised working dilutions of the primer sets. The cycle number at which the reporter signal first increased above the set threshold (Ct value) was plotted against the $\log_{10}$ concentration of input DNA. The data shown are the mean of results from at least 2 independent experiments. Regression lines were fitted to the data; the equation for each line is shown in the figure, as is the correlation coefficient, R^2, value. Error bars indicate one standard deviation above and below the mean.

gene while the IFN-α (non-α4) primers recognizes > 9 IFN-α subtypes (α-1, -2, -5, -6, -7, -8, -11, -13 and a psuedogene) and, as such, record lower Ct values at the same template concentration as there would be nine times as many IFN-α (non-α4) template as there was every IFN-β template.

The IFN-a4 primer set has a different slope value indicating that the PCR efficiency is not the same as that of the reaction carried out with the other IFN primer sets.

The primers were designed to measure levels of mRNA transcript in two-step reverse-transcriptase PCR assays that employed a real-time quantitative RT-PCR mastermix, in which input RNA template would be converted into cDNA by reverse transcriptase and the cDNA levels subsequently amplified by PCR. To confirm that the efficiencies of the primers in the two-step RT-PCR reaction would follow a similar trend to those in the one-step PCR assay, the primers were tested on 10-fold serial dilutions of total RNA extracted from polyIC stimulated BM-DC as polyIC is a known inducer of type 1 IFN. The HPRT and Mx1 primer sets, which could not recognize their respective genes in genomic DNA as the primers go across exon-exon boundaries, were also tested. The Ct values were plotted against the $\log_{10}$ concentration of input total RNA. As shown by the standard curves generated (Figure 3.17), the two-step RT-PCR assays did not have the same efficiency as the one-step PCR assays (Figure 3.16); however, the trends observed in efficiencies of the individual primer sets in the RT-PCR assays mirrored the results obtained when the primer sets were tested with genomic DNA. Again, the IFN-β and IFN-α(non-α4) primer sets had similar slope values, while the IFN-α4 RT-PCR gave rise to a different slope value. The HPRT primer set had a similar efficiency to that of the IFN-β and IFN-α(non-α4) primers while the efficiency of the Mx RT-PCR was slightly different to that of all the other

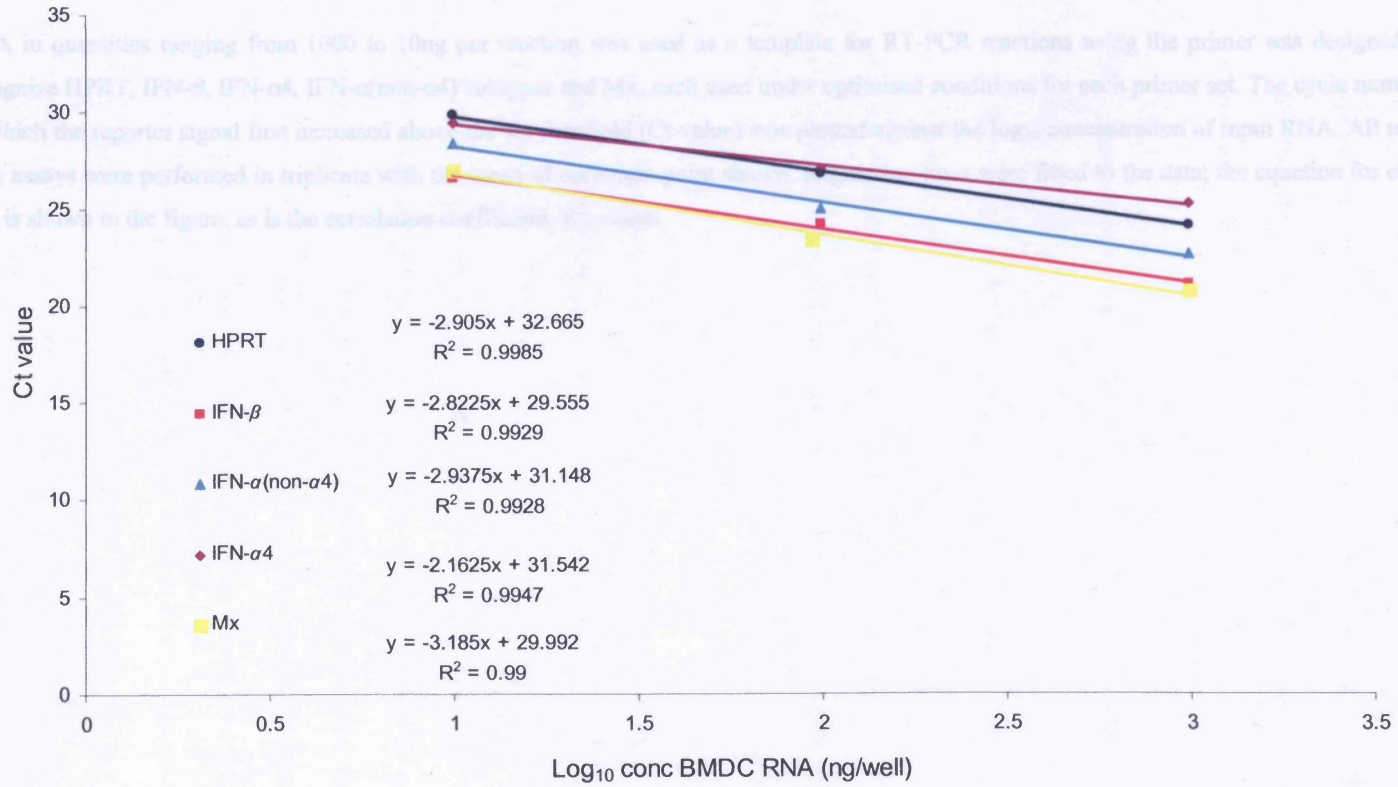

Figure 3.17 Standard curves for real-time RT-PCR analysis of IFN-β, IFNα(non-α4) subtypes, IFN-α4, Mx and the housekeeping gene HPRT performed with serially diluted RNA from polyIC-stimulated BMDCs (legend on following page)

RNA in quantities ranging from 1000 to 10ng per reaction was used as a template for RT-PCR reactions using the primer sets designed to recognize HPRT, IFN-β, IFN-α4, IFN-α(non-α4) subtypes and Mx, each used under optimised conditions for each primer set. The cycle number at which the reporter signal first increased above the set threshold (Ct value) was plotted against the $\log_{10}$ concentration of input RNA. All real-time assays were performed in triplicate with the mean of each data point shown. Regression lines were fitted to the data; the equation for each line is shown in the figure, as is the correlation coefficient, R^2, value.

RT-PCR assays. Although these quantitative RT-PCR assays were suitable for comparison of the levels of a given IFN subtype in different samples, they could not be used for accurate comparison of levels of different IFN subtypes, e.g. IFN-β and IFN-α4 within a sample or between samples.

3.6 Analysis of type 1 IFN levels in FSDC cells after stimulation with viruses or polyIC

After developing and evaluating new and existing methods to measure type 1 IFN mRNA, protein or activity, the most promising methods, namely the real-time quantitative RT-PCR and the EMCV antiviral protection bioassay, which measured type 1 IFN mRNA and activity respectively, were applied in a pilot experiment to verify that the selected, optimized protocols could be successfully employed for analysis of type 1 IFN responses in experimental systems.

A DC cell line, FSDC, was stimulated with 50μg/ml of polyIC. At 4 and 18 hours post stimulation, total RNA was extracted from the cells and levels of type 1 IFN mRNA transcripts (IFN-β, IFN-α4 and IFN-α (non-α4)). Figure 3.18A shows the levels of each mRNA Type 1 IFN subtype induction was induced measured by real-time PCR in samples stimulated with poly IC for different durations while the corresponding type 1 IFN activity present in the supernatant of each sample is displayed in (Figure 3.18B). For the real-time quantitative RT-PCR, the raw data in the form of Ct values for the gene of interest was analysed as discussed in section 2.6.6.2 and the values shown in Figure 3.18A are the ΔCt value which has been normalised against the housekeeping gene HPRT. A ΔCt value that is higher than the ΔCt value of the unstimulated control indicates an increase in the relative levels of the transcript of the gene of interest over the control. Type 1 IFN production was induced in response to

A) Taqman RT-qPCR

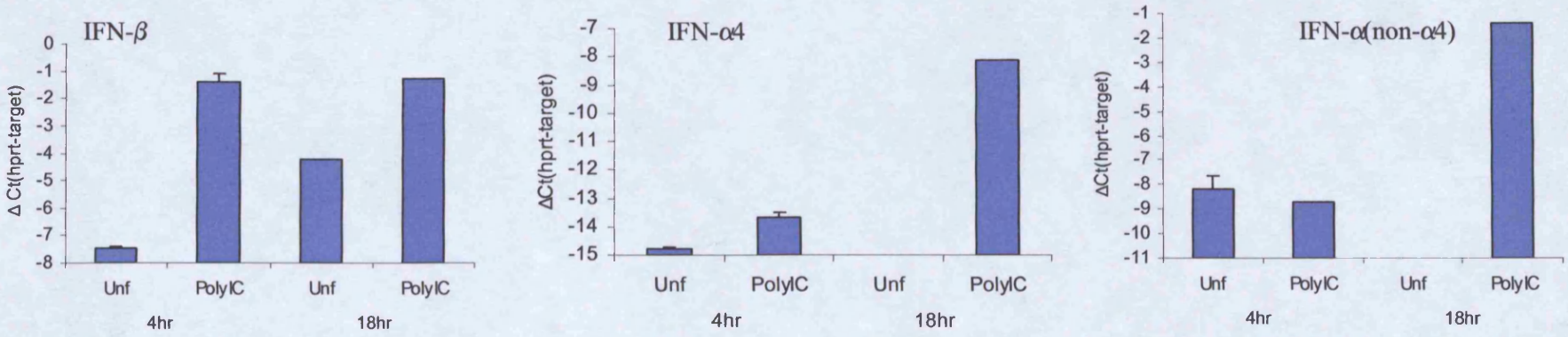

Figure 3.18 A) Quantitation of the levels of IFN-β, IFN-α4 and IFN-α(non-α4) mRNA induced in FSDC cells after stimulation with polyIC

FSDC cells ($1x10^5$/well) were stimulated with 50μg/ml polyIC. At 4 and 18 hours post-treatment, stimulated and unstimulated (unstim) cells were collected, RNA was extracted and levels of individual type 1 IFN mRNA subtypes were determined by Taqman RT-qPCR (A). The level of each target gene was normalised by subtracting the Ct value of the target from the Ct value of the invariant housekeeping gene HPRT of the same sample to obtain the ΔCt value. The results shown are the mean ΔCt value of each sample from two independent experiments, with the error bars indicating one standard deviation (SD) above the mean.

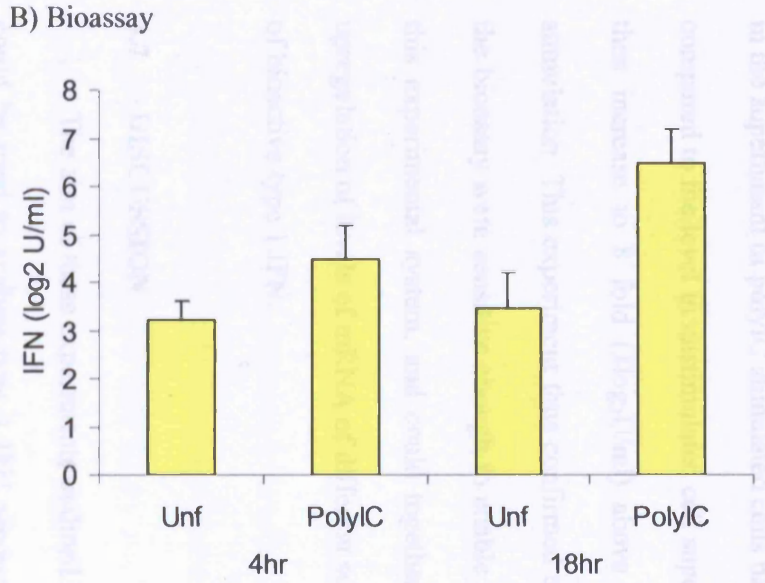

Figure 3.18 B) Quantitation of supernatant type 1 IFN activity induced in FSDC cultures after stimulation with polyIC

FSDC cells ($1x10^5$/well) were stimulated with 50μg/ml polyIC. At 4 and 18 hours post-treatment, supernatants were collected from stimulated and unstimulated (unstim) cells and tested by bioassay to determine the level of type 1 IFN activity present (B). The results are the mean $\log_2$ value from two independent experiments, and the error bars show one standard deviation above the mean.

stimulation of FSDC cells with polyIC. Upregulation of type 1 IFN mRNA levels was detectable at 4 hours and 18 hours post-stimulation. Levels of IFN-β and IFN-α4 subtype mRNA had increased over background unstimulated levels by 4 hours post polyIC treatment. IFN-β mRNA expression remained at the same level at 18 hours post-stimulation, whilst mRNA levels had undergone further increase by this time and expression of IFN-α(non-α4) subtypes continued to be induced. Type 1 IFN activity in the supernatant of polyIC stimulated cells had doubled (and increase of 1 log_2U/ml) compared to the level in unstimulated cell supernatant at 4 hours post-stimulation and then increase to 8 fold (3log_2U/ml) above the control level by 18 hours post-stimulation. This experiment thus confirmed that real-time quantitative RT-PCR and the bioassay were sensitive enough to enable analysis of the type 1 IFN response in this experimental system, and could together provide a picture of the kinetics of upregulation of levels of mRNA of different subtypes of type 1 IFN and of production of bioactive type 1 IFN.

3.7 DISCUSSION

The aim of these experiments outlined in this chapter was to set up assays that could be used to analyse type 1 IFN production during acute and chronic LCMV infection. These assays had to be able to detect low levels of type 1 IFN. Existing assays that measured type 1 IFN protein or activity were evaluated for their sensitivity and utility. In addition, new assays were set up namely reporter gene-based assays for quantitation of murine type 1 IFN activity and real time quantitative RT-PCR assays for the measurement of distinct type IFN subtypes. Following development and evaluation of multiple assay methods, two approaches were chosen for quantitation of type 1 IFN mRNA levels and activity in future experimental work. The chosen assays

were trialed in a pilot experiment to confirm their suitability for evaluation of type 1 IFN responses and the complementarity of the data obtained using two different assays.

A number of systems exist for measurement of type 1 IFN protein levels in biological samples (reviewed in (Meager 2002)). Commercial ELISA kits are available that can not only measure type 1 IFNs but also distinguish between IFN-α subtypes and IFN-β. I found that laboratory and commercially available ELISA kits for quantitation of murine IFN-α were reliable but fairly insensitive, with limits of detection of 25 pg/ml or 50-100 pg/ml respectively. Furthermore, the IFN-α ELISAs only measure the total amount of protein within a sample, and as different interferon subtypes have been reported to have different bioactivity, do not provide information on the units of biological activity of the IFNs. We did not have a chance to evaluate the IFN-β ELISA (RnD systems cat no:42400-1), which was only released in late 2004, which was reported by the manufacturers to have a detection range of 15.6-1000 pg/ml.

Cell based assay systems include the classic antiviral protection bioassay system, where IFNs are detected by their ability to confer an antiviral state on a susceptible fibroblast cell line such as L929 cells and protect it from lysis by a cytopathic virus such as EMCV or VSV (Ishitsuka et al. 1977). This is the system upon which a unit of IFN activity is defined. In my hands, the EMCV antiviral protection bioassay proved to be reliable and to have a reproducible endpoint of ~3.9 or 2 $\log_2$ U/ml with a recombinant IFN-α standard curve, which is almost 100 times more sensitive than the IFN-α ELISA assay. While the bioassay is not able to distinguish between IFN-α, IFN-β or IFN-γ, nonetheless the activity of IFN-α, IFN-β or IFN-γ can be blocked by addition of excess subtype-specific neutralising antibody to the samples before they

are mixed with the cells (Figure 3.2), enabling dissection of the IFN subtypes mediating the observed activity.

Some of the experimental samples to be tested would contain IFN-stimulatory components such as polyIC, CpG, R848 or LCMV. The TLRs expressed by L929 cells is unclear, so the cells were tested for production of endogenous type 1 IFN activity upon exposure to these TLR ligands. The cells were insensitive to all the stimuli at the concentrations tested, apart from LPS which in any case was not used as a test stimuli, which were the maximum concentrations the samples would contain. With LCMV, L929 cells did not exhibit endogenous antiviral protection in the presence of up to 10^6 p.f.u./ml of LCMV Cl13 (Figure 3.3). It was unlikely that LCMV-infected samples would contain more than 10^6 p.f.u./ml of virus and thus there should not be any endogenous antiviral protection by L929 cells in response to either virus or residual type 1 IFN stimuli in the experimental samples I planned to go on to test.

Another cell based assay system involves the use of cells transfected with reporter genes linked to an IFN-responsive promoter (Canosi et al. 1996; Fray et al. 2001; Hammerling et al. 1998; Lewis 1995). Like the classic bioassay system, this differs from the ELISA system in that it quantitates IFN activity, and it has the advantage that this quantitation is based on the level of induction of the reporter gene, which also allows signal amplification, enabling these assays to be very sensitive. The use of promoters responsive only to IFNs gives the reporter gene assay more specificity than the classic bioassay, although until promoters specifically responsive to subtypes of type 1 IFN are identified, subtype specific blocking antiserum must still be used to dissect the IFN subtypes present in a sample. The reporter gene system method is also quicker and easier than the virus inhibition bioassay. The reporter systems I obtained

were based on two different IFN responsive promoters. The ISRE-luciferase reporter (p(9-29 ISRE) 4tkΔ(-39)lucter, (Figure 3.4A) has 4-human ISRE promoter sequences upstream of the luciferase gene (King and Goodbourn 1998) while the MX-CAT (pHH-CAT, Figure 3.4B) plasmid has the promoter for the human MxA gene linked to the gene for CAT. Both promoters respond to both IFN-α and IFN-β and very little if at all to IFN-γ (Baigent et al. 2002) (Figure 3.10). The MX-CAT system has been reported to be able to detect very low levels of human (Ronni et al. 1998) or bovine (Baigent et al. 2002) type 1 IFNs. The ISRE-luc plasmid has been mainly used in human IFN signalling studies where it has been found to be highly inducible by low amounts of IFN (King and Goodbourn 1998).

Type 1 IFNs are not strictly species-specific, rather they have a defined host range (Bridgman et al. 1988). Existing reporter gene systems which were set up in human or bovine systems could not be used to measure murine type 1 IFNs as the IFN-R cell surface receptor did not recognize IFN-α/βs from murine species (my unpublished observations). The promoter sequences of Mx was derived from mouse Mx protein (Lleonart et al. 1990) and the ISRE used, although derived from human genes, would nonetheless be recognized by the activated murine transcription factors. As such, I decided to transfect the reporter plasmids into a murine cell line, in order to allow the murine type 1 IFNs to be recognized by the murine IFN-R and activate the signalling pathway, culminating in the expression of the transfected reporter gene. Transient transfection assays were initially conducted to determine whether transiently-transfect cells would respond to type 1 IFN in a quantitative and reproducible manner.

Previous optimization assays conducted in the laboratory had shown that out of a panel of cell lines tested, which included the fibroblast cell lines BalbCl7 and MC57 and the macrophage-like cell lines IC-21 the highest levels of plasmid uptake

occurred in BalbCl7 cells (Guyver 2001). To increase the transfection efficiencies the transfection protocol was further refined by extensive testing of a range of transfection reagents (FuGene, GeneJuice and Effectene) at different DNA:lipid ratios. When the optimised protocol, which used DNA to Effectene at a ratio of 1:15, was followed to transfect early passage BalbCl7 cells, the efficiency, as measured by GFP reporter expression, was between 50-70% (Figure 3.5). Despite these reasonably good transfection efficiencies, I did not see quantitative responses to IFN-α or IFN-β in these transfected cells. Excessive cell death was observed after transfection, with 50% of the cells typically dying post transfection, drastically reducing the total number of live cells per well, which may have been another one of the factors that contributed to the poor responses observed in this assay. In human signalling studies that employed the ISRE-luc reporter assay, the plasmid was transiently transfected into human 293T cells containing the SV40 T antigen which allows for highly efficient uptake of transfected DNA, so there would be consistent luciferase production in all the wells of transiently transfected cells in a dose dependent manner. However, another, perhaps more significant problem was the high background obtained. This was likely the result of endogenous production of type 1 IFN, which may have been triggered by the transfection procedure itself, in which cells are often incubated overnight with the lupid:DNA mixture in a low serum environment which may have induced cellular stresses, or type 1 IFN may have been induced by the toxicity of the lipid and the presence of dead and dying cells in the population after after transfection. Furthermore, it has been recently reported that type 1 IFN may be upregulated in response to the presence of DNA in the cytosol, such as would have occurred in the transiently-transfected cells (Stetson and Medzhitov 2006).

Given the problems I encountered with the transient transfection approach, I decided to generate cell lines stably transfected with the ISRE-luciferase and MX-CAT constructs in order to simplify the assay and make the results more reproducible. I used both constructs because the MX-CAT reporter system in bovine cells had a reported sensitivity of 0.32U/ml (Fray et al. 2001) and I hoped it would prove to be similarly sensitive in detecting murine type 1 IFNs, but the luciferase reporter system appeared to offer advantages in terms of ease, speed and cost of the reporter assay.

Both plasmids did not have a selectable eukaryotic marker and so they were co-transfected with a plasmid encoding blasticidin resistance and transfected cells were selected based on blasticidin resistance. Initial tests using the pooled blasticidin resistant cells showed that quantitative responses against recombinant human and murine IFNα and IFN-β could be generated using the ISRE-luc system. However, with increasing passages, the BalbC17 ISRE-luciferase cells became less sensitive to type 1 IFN, as the background continued increasing, necessitating sub-cloning. Although I was able to generate subclones that reproducibly gave a dose-dependent response to concentrations of IFN-α and -β between 10U/ml and ~500U/ml (Figure 3.10), and successfully used these in preliminary experimental work, assays conducted using these stably transfected cells suggested they were not as sensitive to low type 1 IFN concentration as hoped.

A drawback to the reporter gene-based IFN assays is that the promoters are responsive to both IFN-α and IFN-β, hence they do not differentiate the subtypes of type 1 IFN present in a given sample. To solve this problem, I attempted to find antibodies that would specifically neutralise IFN-α and IFN-β activity, allowing each subtype to be assayed in isolation. Neutralizing antibodies against murine IFN-α and

IFN-β were tested and appear to work (Figure 3.11) specifically neutralising the activity of the appropriate type 1 IFN subtype.

Although the reporter gene-based assays initially worked reasonably well, I ultimately made a decision not to use them because I found they lost their sensitivity to IFN-α after several passages in culture. (Figure 3.12). The background luciferase expression levels increased, resulting in the cells beoming less sensitive to lower doses of IFN-α. Efforts were made to reduce the endogenous background luciferase expression levels, for example, background levels generally increased when unstimulated cells became confluent, and so the cell numbers were halved from $2x10^4$ to $1x10^4$ cells/well in the 48-well plate format to reduce the chance of the cells becoming confluent and maintain the sensitivity of their response to IFN-α. It was not clear what caused the increasing background levels. The plasmids may have integrated or been maintained episomally. If the plasmids were integrated into genomic DNA, chance integration into a transcriptionally active site may have resulted in high, non-specific luciferase expression; this has been observed in other laboratories which attempted to generated stably-transfected reporter systems using the ISRE-luc vector (S.Goodbourn, personal communication).

Other reporter gene assays circumvented this problem by utilizing cells which had lost their IFN genes e.g. Vero cells, which respond to human but not murine IFN-α/β, or by extensive testing of transfected clones for one with the best signal to noise ratio such as was conducted when developing transfected Mx-CAT MDBK cells (B. Charleston, personal communication). I tested 30 and 64 clones for ISRE-luc and Mx-CAT inducible activity respectively and selected the best responders, which worked initially before losing their sensitivity over extensive passages. If I had more time, I could have tested more clones, but there were other objectives that needed to be met

and we had set up and evaluated the protocol for the EMCV bioassay in the meantime and decided to use this method for analysis of experimental samples. Human cell lines which have a targeted disruption of the IFN-locus and are thus unable to produce type 1 IFN have recently been described (Young et al. 2003) and an analogous system set up in murine cells could prove to be a suitable carrier cell line to develop such assays in the future.

In future experiments, I felt it would be of interest to dissect the subtypes of type 1 IFN produced by different cell types/tissues and at different stages of infection, as there is increasing evidence that their production is temporally regulated. For example, IFN-β and IFN-α4 are thought to be more important than the other -α subtypes during the initial stages of infection, and to trigger additional IFN induction (Capobianchi et al. 2003; Deonarain et al. 2004). There is also evidence that IFN-α subtypes are differentially regulated, with IFN-α2, -α5, -α6 and –α8 reported to be specifically upregulated during viral infections (Marie et al. 1998), reviewed in (Levy et al. 2003).

As none of the protein/activity-based assays I had established/evaluated had the capacity to distinguish subtypes of IFN-α to allow more detailed analysis of the kinetics of production of different IFN subtypes, real-time quantitative RT-PCRs were thus set up to detect specific IFN subtype transcripts. Conventional RT-PCRs primers specific for murine IFN-β and discrete IFN-α subtypes have been designed (Deonarain et al. 2000). However, we found these conventional RT-PCRs to be semi quantitative and not sensitive (data not shown). Real-time PCR assays which rely on the release of fluorescent products during the amplification phase of a PCR reaction to measure levels of the transcript of interest have been demonstrated to be sensitive

than conventional PCR assays and may posses a linear detection range of up to 7 (or 5) logs .

Real-time quantitative RT-PCR analysis of IFN mRNA also complements the bioassay system by enabling detection of IFN responses in samples that can't be tested in the bioassay, e.g. tissue samples.

The major difficulty in designing primers that were specific for a given type 1 IFN subtype lay in the fact that they shared close homologies with one another. While the IFN-β gene only shared 30-40% homology with the IFN-α subtypes, the 12 IFN-α subtypes, whose genes are located in a cluster on chromosome 4, have a homology of ~80% to one another (De Maeyer and De Maeyer-Guignard 1998). Heterologous regions within the genes, which could be used to distinguish the IFN-α subtypes, were limited. To compound matters, the real-time RT-PCR assay sets a fixed annealing temperature and elongation period for the PCR amplification cycles, so all real-time Taqman primers and probes must be designed to be optimal within the set values. This is not the case for conventional PCRs, where the annealing temperatures and elongation periods are customised to the primers. In addition, primers for real-time Taqman RT-PCR assays are subject to many constraints, which are listed in Section 2.6.1. These were adhered to by the Primer Express software package that was used to predict suitable primer-probe sets for the genes. Among the constraints listed for the probe sequence are that the number of cytoseine (C) residues must exceed the number of guanine (G) residues; furthermore, the primer sequence should not have more than 2 C/G residues in the last 5 nucleotides at the 3' end. As the all the IFN-α/β sequences are posses GC-rich regions as observed by ClustalW alignment, this severely limited the number of heterologous regions of sufficient length that encoded sequences which were suitable for the design of real-time RT-PCR primers and probe.

Taqman primer sets for IFN-β and IFN-α4 had already been described (Karaghiosoff et al. 2003); these were re-checked to confirm that they were the optimal primers for their respective genes. The IFN-α4 primer set generated a less efficient RT-PCR than to the other primer sets. However, I did not change this primer set in favour of more efficient alternatives as the forward primer of this set spanned a 15bp deletion within the IFN-α4 gene which did not occur in the other IFN-α subtypes, thus ensuring the reaction would be specific for IFN-α4 only (Figure 3.14). Ideally discrete primers and probes would have been designed for all IFN-α subtypes. However, in the face of the severe constraints on primer design, we could only design a primer and probe set that recognized a region identical in all the IFN-α subtypes apart from IFN-α4 in order to study the induction of these subtypes. Real-time quantitative RT-PCR assays were conducted on an ABI-SDS instrument, which also supported assays based on the Sybr-Green detection system. While the cyber-green assay could have been applied and indeed have since been developed to detect IFN-α/β transcription (Honda et al. 2005; Sato et al. 2000), we decided to use the Taqman real-time quantitative RT-PCR system as there was the extra specificity afforded by the binding of the probe sequence. This would potentially reduce the detection of genomic DNA contamination, as it lengthened the template that needed to be recognized and was thus less likely to bind to fragments of genomic DNA which might still be present after DNase treatment. Furthermore, it has been suggested that Sybr-Green PCRs may be less sensitive than assays employing the fluorogenic probe (Yin et al. 2001).

Real-time quantitative RT-PCRs for IFN-β, IFNα-4, IFN-α(non-α4) and Mx were set up (Deonarain et al. 2004; Montoya et al. 2002) and optimized (Figures 3.14 to 3.17). Type 1 IFN mRNA are low abundant transcripts because until their production is stimulated by the presence of infections, there is usually very little type 1 IFN

produced by a resting cell *in vivo*. It was therefore crucial that the IFN α/β real time PCR assays were not only specific but also sensitive. Several 3' labelling and modifications are available which are designed to improve the specifity and sensitivity of the binding of the probe to the PCR amplicon. One of these modifications is the attachment of the non-fluorescent minor groove binder (MGB) to the 3' end of the probe. MGB is a non-fluorescent conjugate which forms hyper stabilized duplexes with complementary DNA enabling MGB-conjugated probes to hybridize with greater sequence specificity than, it is claimed, natural unconjugated probes(Afonina et al. 2002; Kutyavin et al. 2000; Letertre et al. 2003). Conventional FAM-TAMRA and MGB labelled probes for IFN α4 and nonα4 were synthesised and compared - we found in our hands that the MGB-labelled IFN-α4 probe had an improved the efficiency of its real-time RT-PCR over the unconjugated probe. Probes for the IFN-α4 and IFN-α(non-α4) RT-PCR assays were thus synthesised with the MGB modification. The MGB-probe and primers were shown to be specific for the IFN-α4 subtype as it did not amplify any of the other closely-related α subtypes (Figure 3.15).

The reliability of a quantitative real-time RT-PCR assay is dependent upon several elements, including the treatment/quality of the input RNA, the intra-assay variation, the selection of the housekeeping gene and the normalisation strategies employed. The quality of the input total RNA was a key concern throughout the project as the interferon genes do not posses introns (De Maeyer and De Maeyer-Guignard 1998) and so the primers would not be able to discriminate between RNA transcripts and genomic DNA contamination. I attempted to minimize the likelihood of genomic DNA contamination by treating all samples with DNase prior to quantitative RT-PCR analysis. The intrassay variation was tested by using known concentrations of

genomic mouse DNA as template and was found to be minimal (unpublished observations). Type 1 IFN transcripts are not normally highly abundant in the cells, and so the housekeeping gene selected should also be of similar low abundance in order to ensure any discrepancies in the amount of input sample is not masked by the saturating levels of the housekeeping gene. The HPRT gene was used as the housekeeping gene because it was normally present in low levels in cells, and partly because it had been previously used as the housekeeping gene in the quantitative analysis of IFN-α4 and IFN-β (Karaghiosoff et al. 2003). An ideal housekeeping gene should have an invariant rate of expression under any situation, and in this regard, HPRT qualifies as its expression does not show much variation under different conditions (Loseke et al. 2003). However, it may have been more appropriate use β-glucoronidase as a housekeeping gene as it displayed more stable expression, or to use a panel of housekeeping genes as control for one another.

There are several ways to normalise the results (in Ct values) of real-time quantititative PCR assays. Common methods include running a standard curve of plasmid containing a known copy number of the target gene alongside the unknown samples, which would express the results as mRNA copy number per sample; or by expressing the level of a target gene in a treated sample as the fold difference over the level in a control sample (Pfaffl 2001). Quantitation by standard curve analysis did not prove practical for the large number of samples involved as a standard curve would have to be run on every plate that was tested. Baseline levels of IFN-β and IFN-α in uninfected unstimulated control samples were usually very low or non-existent, and as such I felt it was not valid to express the levels as fold induction over either the baseline or an arbitrarily selected sample expressing the gene. I also decided that the fairest way to determine the levels of IFN-α and β in the different samples

would be to analyse expression levels in a fixed amount of sample. As such, all the samples were quantitated by UV spectroscopy after RNA extraction the concentration of input RNA was fixed at 300ng/well for all samples where possible. In some cases, such as during the isolation of mRNA from Mo-Flo sorted dendritic cells, there was not enough RNA for quantitation, and there, RNA was extracted from an equal number of cells from each subpopulation in every sample in each specific experiment. The levels of HPRT were checked to ensure they were similar in all the samples tested. Furthermore, the results were presented with minimal manipulation of the raw data, which was normalised by subtracting the Ct value of the target gene from the Ct value of the housekeeping gene, thus, the larger the difference in the Ct value (ΔCt), the higher the mRNA level of the target gene in the sample.

Having evaluated a range of IFN-α/β-quantitation assays, I selected the EMCV antiviral bioassay as the best means to measure secreted bioactive IFN levels and the real-time quantitative RT-PCR to measure cellular levels of type 1 IFN mRNA and to discriminate between induction of IFN-β, IFN-α4 and the remaining IFN-α subtypes. The EMCV antiviral protection bioassay was demonstrated to be sensitive and consistent. The main weakness of this assay is that there are other cytokines, e,g. TNF-α that may be present in experimental samples and which may exert anti-viral effects in this assay (Jacobsen et al. 1989); however, this drawback can be overcome using blocking antibodies to demonstrate that antiviral activity in experimental samples is indeed mediated by IFN-α/β. Another consideration was that the samples would likely contain IFN-inducing stimuli such as LCMV or TLR ligands, which may possibly induce endogenous type1 IFN infection in the L929 cells. Tests conducted to determine the potential of this effect showed that L929 cells do not secrete endogenous type 1 IFN in response to LCMV unless it was present at very high titres

not likely to occur in our samples and did not respond polyIC, R848 or CpG DNA that would be used as stimuli.

The assays were applied in a pilot experiment to determine whether the data obtained by two different methods would concur with each other. A murine DC cell line, FSDC (Girolomoni et al. 1995) was stimulated with poly IC, a synthetic ligand of TLR3 known to induce type 1 IFN production in DCs. Quantitation of type 1 IFN mRNA levels at two early timepoints after stimulation indicated that IFN-β and IFN-α4 were the subtypes initially most strongly upregulated, with the expression of the other IFN-α subtypes only increasing at the later timepoint of 18 hours post stimulation, by which time IFN-β expression had reached a plateau. Expression of IFN-α4 continued to increase between 4 and 18 hours post stimulation. Low pre-existing levels of all type 1 IFN subtypes were detected in unstimulated controls. The EMCV bioassay confirmed the presence of low levels of type 1 IFN production by unstimulated cells, as the assay detected low type 1 IFN bioactivity of about 3 $\log_2$ units/ml in the cell supernatant (Figure 3.18B). Results from the EMCV bioassay were also consistent with the observations made at the mRNA level, in that a small increase of IFN activity was detectable in the supernatant at 4 hours post-stimulation, and higher type 1 IFN activity levels were observed by 18 hours post-stimulation. Previous reports indicate that upon stimulation/infection, fibroblast cells predominantly secrete IFN-β and IFN-α4, which then signals through the IFN-R to upregulate expression of the other IFN-α subtypes (Goodbourn et al. 2000). These results suggest that type 1 IFNs may be induced in a similar manner in these DC cell lines upon polyIC stimulation. It has been previously reported that infection of D2SC/1 and FSDC with live or inactivated virus induced higher levels of IFN-β activity than IFN-α (Eloranta et al. 1997) and our data, showing early upregulation of IFN-β which was sustained still sustained 18

hours post-stimulation does lend support to this observation. The results of the pilot experiment indicate that both the assays are reliable and are able to complement each other.

These primers have since been used to measure the levels of type 1 IFN mRNA induced in response to i.p. infection with LCMV Arm in the spleens of mice expressing different isoforms of CD45 or lacking the molecule entirely (Montoya et al. 2006). CD45 knock-out mice were found to mount only a partial type 1 IFN response against the infection – while IFN-α4, IFN-α(non-α) and IFN-β transcripts were detected in the spleens of these mice post-infection, the levels were lower than responses recorded in wild-type mice or transgenic mice that expressed one of the isoforms of CD45. Furthermore, the results of the real-time quantitative RT-PCR analysis were in agreement with data of the serum type 1 IFN levels, which was measured by IFN-α ELISA, which showed that serum IFN-α levels in the infected CD45 knock-out mice were also reduced. These, plus observations from other experiments does suggest that CD45 is required for normal type 1 IFN responses to viral infection.

Since the time my project was initiated, other type 1 IFN quantitation protocols have reported, namely a reporter gene-based bioassay utilizing GFP expression (Bollati-Fogolin and Muller 2005), while the company Applied Biosystems now offer pre-designed, pre-optimized Taqman real-time quantitative PCR assays which the manufacturers claims is subtype specific, with individual assays for IFN- β and α1, 2, 4, 5, 6, 7, 9, 12 or 13 subtypes available as inventoried or made-to-order assays (Applied Biosystems, product.appliedbiosystems.com). However, the existing issues which I encountered during development of the reporter gene-based bioassay and real-time quantitative RT-PCR assays have not all been overcome by the reported

methods. For example, the newly published eGFP bioassay is not very sensitive, as the detection range was reported to be between 20-300U and 10-500U for IFN-α and IFN-β respectively and it is not able to distinguish between the activity of IFN-β and different IFN-α subtypes. The sensitivity and specificity of the commercially available Taqman assays was not reported by the manufacturer (indeed, some of the assays have yet to be assessed by quality control) and all cannot exclude detection of the genomic sequence. Furthermore, no information is provided as to whether the readings of the assays could be compared with each other. Nonetheless, given the opportunity, it would have been interesting to compare the sensitivity of some of the IFN real-time assays I used, especially the IFN-α4 subtype assay, in order to determine if the commercially available assay possessed a higher level of sensitivity/efficiency than the assay I employed.

3.8 Conclusions

Methods were set up and optimized to allow sensitive, reliable measurement of type 1 IFN protein and mRNA levels in *in vivo* and *in vitro* samples. In addition to evaluating established protocols for detection of type 1 IFNs at the protein level such as the IFN-α ELISA and the EMCV antiviral bioassay, new assays based on reporter gene technology were developed, optimized and tested. Early in development, reporter gene based-assays were more sensitive than IFN-α ELISAs, and were equally or slightly more sensitive than the bioassay. However, the reporter cells gradually lost their sensitivity to type 1 IFN as they were passaged, so this approach could not be used as a quantitative assay in future studies. It was thus decided to use the EMCV bioassay to determine the levels of bioactive type 1 IFN in the serum or supernatants. Real-time quantitative RT-PCR assays were also set up that measured transcript levels

of IFN-β, IFN-α4 and other IFN-α subtypes to enable information on the mRNA levels of different IFN subtypes in tissues and cells to be obtained.

A pilot experiment tracking type 1 IFN induction in a DC cell line after polyIC stimulation confirmed that both assays were sensitive and robust enough to be used in the quantitation of type 1 IFNs in future work.

CHAPTER 4

CHARACTERIZATION OF TYPE 1 IFN RESPONSES DURING *IN VIVO* ACUTE AND PERSISTENT VIRAL INFECTIONS

4.1 Introduction

The outcome of a murine LCMV infection in adult, immunocompetent C57BL/6 mice is dependent upon the virus strain employed, the dose and the route of inoculation (Borrow and Oldstone 1997). For example, adult, immunocompetant mice infected i.p. with a low dose (~5 x 10^5 p.f.u) of the Armstrong strain of LCMV will experience an acute infection that is cleared in 7-10 days while i.v. inoculation with a high dose (~5x10^6 p.f.u) of LCMV Cl13 or Docile results in a long-term, systemic infection with high viral loads. Persistent infection also results when mice are infected at birth due to clonal deletion of virus-specific T-cells by mechanisms involved in the induction of self-tolerence. There are a number of differences in the host immune response in LCMV infections that are cleared and infections that go on to persist which may contribute to and/or result from viral persistence (Borrow 1997).

Among the cytokines produced early in infection are type 1 IFNs which both have direct anti-viral effects and important immunoregulatory roles in the innate and adaptive immune responses. Many studies have reported that infection with acute strains of LCMV elicits production of type 1 IFN early in infection (Bukowski et al. 1983; Jacobson et al. 1981; Leist et al. 1987). Biron *et al* report that during LCMV infection, type 1 IFN production in response to the presence of the virus is not high, but this is subsequently amplified through the autocrine amplification via type 1 IFN-R and JAK-STAT signalling (Malmgaard et al. 2002). Type 1 IFNs play a key role in control of LCMV infection (Moskophidis et al. 1994; Ou et al. 2001). However, it is

not clear whether this is a consequence of the direct antiviral effects on limiting viral replication and/or a result of its direct or indirect effects on LCMV specific T-cell expansion. Induction of type 1 IFNs are critical for the proper maturation of LCMV-specific T-cells which that are responsible for controlling and ultimately killing the virus (Bartholdy et al. 2000; Buchmeier et al. 1980; Havenar-Daughton et al. 2006; Ou et al. 2001; Wille et al. 1989). While T-cell exhaustion occurs rapidly in the absence of type 1 IFN activity, this could either be a consequence of increased viral load thus affecting the T-cell response or impairment of the T-cell activity.

While almost any cell type can produce type 1 IFN in response to a viral infection, studies with other viruses (Cella et al. 2000; Dalod et al. 2003; Hochrein et al. 2004; Schmidt et al. 2005) have suggested that the majority of type 1 IFN in the serum is secreted by plasmacytoid DCs in the spleen. Plasmacytoid DCs do not appear to be the predominant source of serum type 1 IFN during acute infection with LCMV as mice which were depleted of plasmacytoid DCs produced similar levels of type 1 IFN as wild-type mice upon infection with LCMV Arm (Dalod et al. 2002). The cells responsible for type 1 IFN upregulation during acute LCMV infection have not been comprehensively defined, although our group has previously reported that the $CD11c^+$ $B220^+$ /$B220^-$ DC subsets within the spleen become highly activated and the $CD11c^+$ $B220^+$ subset produces high transient levels of type 1 IFN upon infection with LCMV Arm (Montoya et al. 2005). Nonetheless, it is unclear if the bulk of type 1 IFN is produced by infected cells or by uninfected cells that sense the presence of viral components. Type 1 IFN production may be triggered by activation of the TLRs on cells that recognize viral components such as viral glycoproteins and nucleic acid, in which case the cell need not necessarily be infected in order to respond, or by the intracytoplasmic helicase pathways that sense viral dsRNA, in which the cells would

have to be infected by the virus and accumulating replicative intermediates in the cytoplasm.

During a persistent infection with LCMV, very high levels of viral stimuli are present systemically for extended periods in the host. Little is known about the regulation of type 1 IFN production in chronic LCMV infection, although old studies have suggested that mice persistently infected at birth with LCMV have reduced levels of circulating serum type 1 IFN and produce lower levels of the cytokine when stimulated with polyIC or Newcastle Disease virus compared to uninfected mice (Holtermann and Havell 1970). However, these studies only analysed type 1 IFN levels during the chronic stage of infection using mice that were infected at birth with LCMV.

In short, the type 1 IFN response during persistent viral infection, the type of cells that produce type 1 IFN during persistent viral infection and the functional state of the innate immune system during persistent LCMV infection still requires investigation. As such, I plan to characterise the type 1 IFN response during persistent LCMV infection by employing the more sensitive quantitative real-time RT-PCR assay to measure levels of type 1 IFN subtype mRNA in spleen of acute and persistently infected mice to determine the levels of induction of individual type 1 IFN subtypes over time. The levels of type 1 IFN activity in the spleen will be measured in parallel using the EMCV bioassay. Furthermore, the specialised type 1 IFN producing cell, the DC population, will also be investigated in order to determine the role that they play in type 1 IFN production during acute and persistent LCMV infection. Immunostaining of spleen sections from acute and persistently-infected mice will also be conducted to gain insight into whether type 1 IFN is predominantly produced by uninfected or LCMV-infected cells. Finally, I also intend to use the quantitative real-

time RT-PCR assays and the EMCV bioassay to analyse the ability of the persistently infected mice to respond to exogenous type 1 IFN inducing stimuli in the presence of high levels of infectious virus.

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4.2 Generation of mice acutely and persistently infected with LCMV

My first goal was to establish an infection regime that would allow the generation of a persistent infection in adult, immunocompetent C57Bl/6 mice. In C57Bl/6 mice infected i.v. with LCMV, the virus replicates systemically and infectious virus can be detected in the spleen and blood at very early times post infection. Whether or not the infection is cleared depends on the infecting virus strain and the dose used (reviewed in (Borrow and Oldstone 1997). Mice were infected i.v. with a moderate dose of LCMV Arm, a strain that is cleared even when administered i.v. at a high dose to adult mice, or with two different LCMV strains previously reported to cause a persistent infection in adult mice, LCMV Cl13 at $5x10^6$ p.f.u or LCMV Docile, at a range of doses from $1x10^6$p.f.u 50 $5x10^6$ p.f.u. Virus clearance or persistence was then evaluated by bleeding from the tail vein at different times post-infection and determining serum viral titres by plaque assay (Arm or Cl13) or immunoplaque assay (Docile).

4.2.1 Serum viral titres in LCMV-infected mice over time

A comparison of the levels of infectious LCMV in the serum after i.v. inoculation with three different LCMV strains is shown in Figure 4.1. None of these strains caused a lifelong

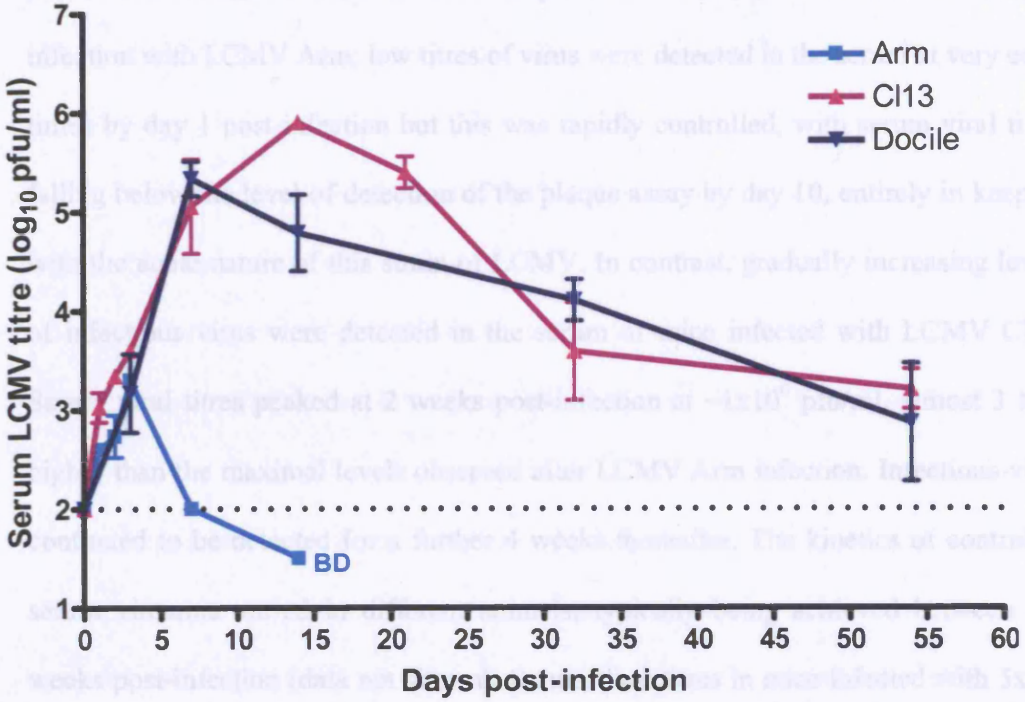

Figure 4.1 Kinetic analysis of serum viral titres in mice infected with LCMV Arm, Cl13 or Docile

C57BL/6 mice were infected i.v. with $5x10^5$p.f.u LCMV Arm or $5x10^6$p.f.u LCMV Cl13. At the indicated times post-infection, blood samples were collected from groups of three mice infected with each virus, and serum viral titres of individual animals were determined by plaque assay on Vero cells. The values shown are the mean serum viral titres of each group of mice, expressed as log_{10} plaque forming units (p.f.u)/ml serum. The error bars indicate 1 standard deviation above and below the mean. The assay detection limit is indicated by the dotted line. BD indicates that virus was below the levels of detection at the indicated timepoint. The results are representative of findings made in 2 independent experiments.

persistent infection in adult immunocompetent mice at the doses used here. After infection with LCMV Arm, low titres of virus were detected in the serum at very early times by day 1 post-infection but this was rapidly controlled, with serum viral titres falling below the level of detection of the plaque assay by day 10, entirely in keeping with the acute nature of this strain of LCMV. In contrast, gradually increasing levels of infectious virus were detected in the serum of mice infected with LCMV Cl13. Serum viral titres peaked at 2 weeks post-infection at ~$1x10^6$ pfu/ml, almost 3 logs higher than the maximal levels observed after LCMV Arm infection. Infectious virus continued to be detected for a further 4 weeks thereafter. The kinetics of control of serum vireamia varied in different animals, typically being achieved between 6-8 weeks post-infection (data not shown). Serum viral titres in mice infected with $5x10^6$ p.f.u of LCMV Docile were similar to those in mice infected with LCMV Cl13, peaking at 2 weeks post-infection and at levels approximately 3 logs higher than the peak serum viral titre in mice infected with LCMV Arm, and with virus persisting 8-12 weeks before the infection was ultimately cleared by the host. The duration of persistence after infection with LCMV Docile was wholly dependent upon the initial dose administered, with inoculation of lower doses resulting in earlier clearance of the virus from serum (data not shown). As there was little difference between the duration of virus persistence in mice infected with LCMV Cl13 and LCMV Docile, LCMV Cl13 was employed as the persistent strain for experimental work. It was chosen because it could be compared against LCMV Arm, with which it shared a virtually identical viral sequence aside from a single amino acid substitution in the GP and the RNA-dependent polymerase (Salvato 1993). Furthermore, viral titres could be determined using conventional plaque assays rather than the cumbersome immunoplaque assays required for determining titres of LCMV Docile. In subsequent

experimental work, a persistent LCMV infection was generated by i.v. inoculation of mice with $5x10^6$ p.f.u. of LCMV Cl13.

4.2.2 Viral titres in the spleen of LCMV-infected mice over time

Figure 4.2 shows the levels of infectious virus in the spleen during the course of infection of mice with LCMV Arm or LCMV Cl13. Following i.v. infection with $5x10^5$ p.f.u. of LCMV Arm, infectious virus was detectable in the spleen for 14 days, with peak viral titres in the spleen being observed at day 2 post-infection. In contrast, the level of infectious virus in the spleen of mice infected with $5x10^6$ p.f.u of LCMV Cl13 continued to increase until day 4 post-infection and peak splenic viral titres were approximately 100 times higher than those in mice acutely infected with LCMV Arm. Viral titres in the spleen of the persistently infected mice began to drop between days 4 and 6 post-infection, but here, a moderately higher viral load was still sustained for 4 weeks post-infection, with infectious virus falling below detectable levels in the spleen by 8 weeks post-infection.

4.3 Analysis of type 1 IFN mRNA levels in the spleen during LCMV Arm and LCMV Cl13 infection

Having established an LCMV strain and dose that could be used to generate persistently infected mice (albeit not a lifelong persistent infection), and also analysed the infectious viral titres in the serum and tissues during acute and persistent LCMV infections, I next examined the host type 1 IFN response over the course of an acute infection and one that went on to persist. Mice were infected i.v. with $5x10^5$ p.f.u of LCMV Arm, (to generate an acute LCMV infection) or $5x10^6$ p.f.u of LCMV Cl13 (to generate a persistent LCMV infection), and total

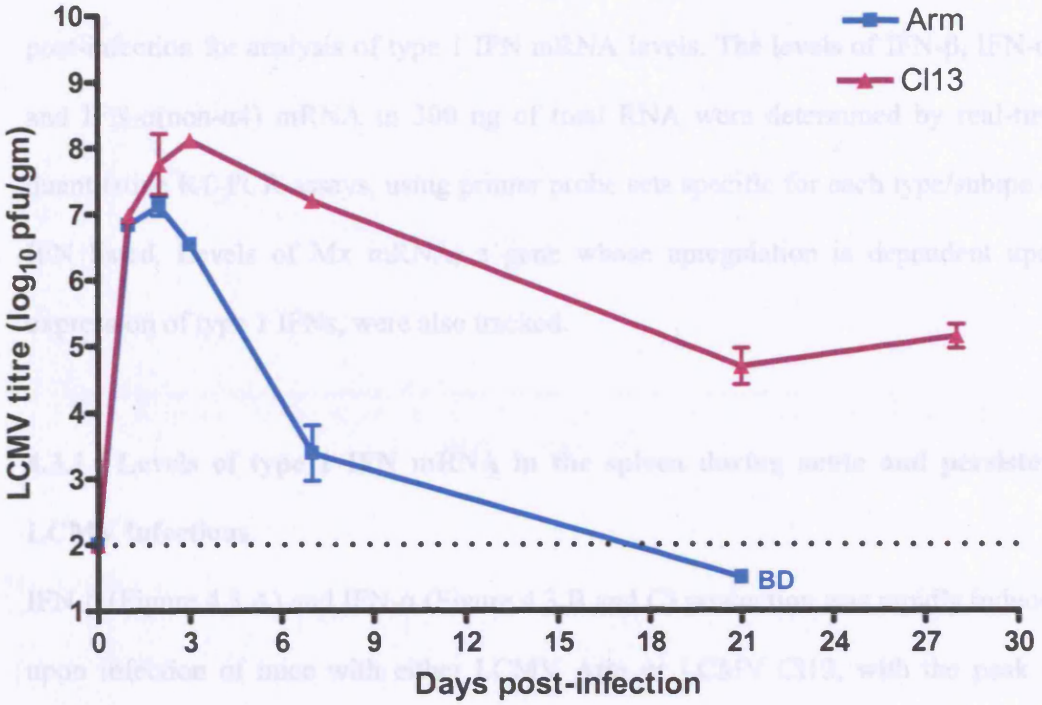

Figure 4.2 Kinetic analysis of viral titres in the spleen in mice infected with LCMV Arm or Cl13

C57BL/6 mice were infected i.v. with $5x10^5$ p.f.u LCMV Arm or $5x10^6$ p.f.u LCMV Cl13. At the indicated timepoints post-infection, spleen samples were collected from groups of three mice infected with each virus, and spleen viral titres were determined by plaque assay on Vero cells. The values shown are the mean spleen viral titres, expressed as log_{10} plaque forming units (p.f.u)/gm spleen. The error bars indicate 1 standard deviation above and below the mean. The assay detection limit is indicated by the dotted line. BD indicates that virus was below the levels of detection at the indicated timepoint. The results are representative of findings made in 2 independent experiments.

RNA was purified from homogenized pieces of snap-frozen spleen at different times post-infection for analysis of type 1 IFN mRNA levels. The levels of IFN-β, IFN-α4 and IFN-α(non-α4) mRNA in 300 ng of total RNA were determined by real-time quantitative RT-PCR assays, using primer probe sets specific for each type/subtpe of IFN listed. Levels of Mx mRNA, a gene whose upregulation is dependent upon expression of type 1 IFNs, were also tracked.

4.3.1 Levels of type 1 IFN mRNA in the spleen during acute and persistent LCMV infections

IFN-β (Figure 4.3 A) and IFN-α (Figure 4.3 B and C) production was rapidly induced upon infection of mice with either LCMV Arm or LCMV Cl13, with the peak of expression occurring at 12-24 hours post-infection in both infections. In mice acutely infected with LCMV Arm, type 1 IFN mRNA levels fell sharply after the peak and returned close to baseline between days 3 and 7 post-infection (as the infection was controlled). The kinetics of the acute-phase type 1 IFN mRNA response in LCMV Cl13-infected mice were very similar to those in mice infected with LCMV Arm, although the peak levels of all type 1 IFN mRNAs were slightly higher in the Arm-infected mice. Notably, the sharp drop in type 1 IFN mRNA levels that was observed in LCMV Arm-infected mice at 48 and 72 hours post-infection also occurred in LCMV Cl13-infected mice, as did the subsequent further decline in type 1 IFN mRNA levels from 3-7 days post-infection, despite the fact that viral titres in the LCMV Cl13 mice were increasing over this time-frame (Figure 4.1 and Figure 4.2). However, there was a difference in type 1 IFN mRNA levels in mice inoculated with LCMV Arm and LCMV Cl13 at later times post-infection: whereas no IFN-β or IFN-α4 mRNA could be

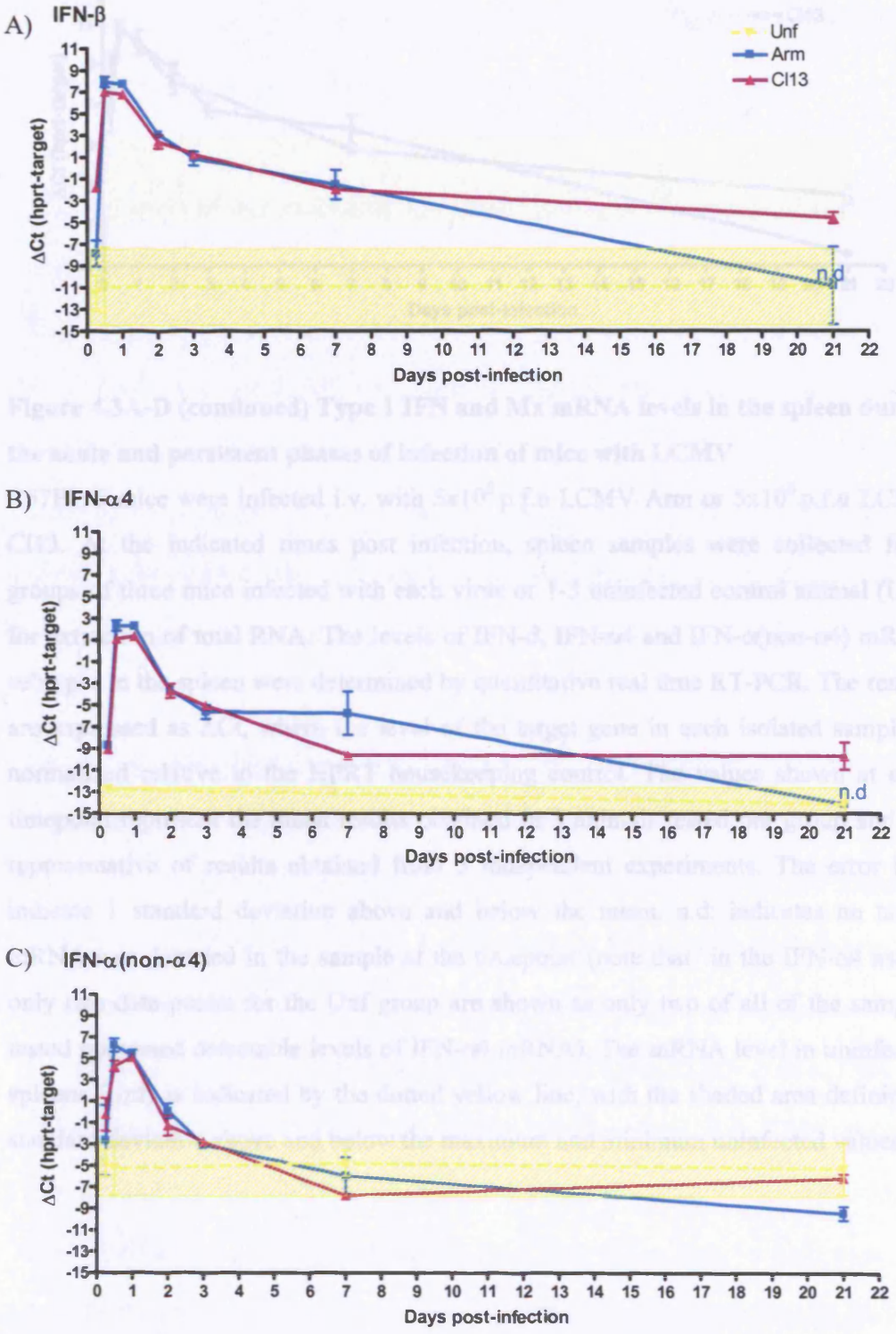

Figure 4.3 A-D Type 1 IFN and Mx mRNA levels in the spleen during the acute and persistent phases of infection of mice with LCMV (figure continued on the following page)

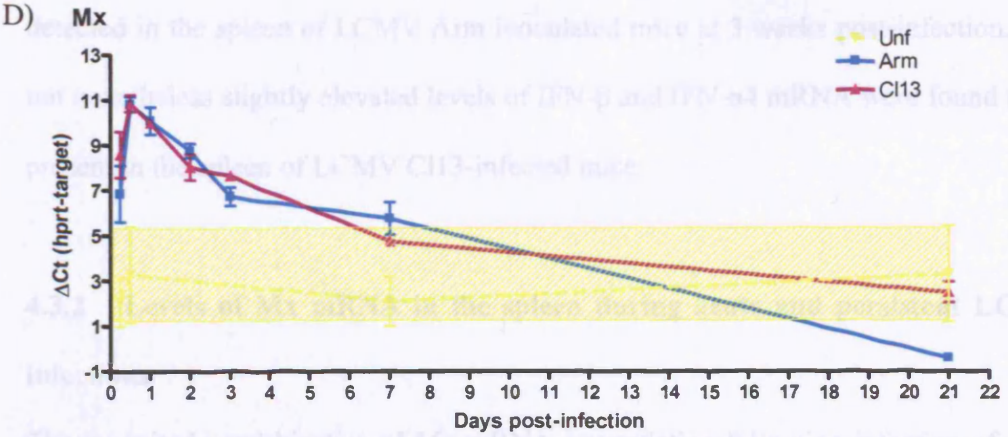

Figure 4.3A-D (continued) Type 1 IFN and Mx mRNA levels in the spleen during the acute and persistent phases of infection of mice with LCMV

C57BL/6 mice were infected i.v. with $5x10^5$ p.f.u LCMV Arm or $5x10^6$ p.f.u LCMV Cl13. At the indicated times post infection, spleen samples were collected from groups of three mice infected with each virus or 1-3 uninfected control animal (Unf) for extraction of total RNA. The levels of IFN-β, IFN-α4 and IFN-α(non-α4) mRNA subtypes in the spleen were determined by quantitative real time RT-PCR. The results are expressed as ΔCt, where the level of the target gene in each isolated sample is normalized relative to the HPRT housekeeping control. The values shown at each timepoint represent the mean results obtained in 3 animals tested per group and are representative of results obtained from 3 independent experiments. The error bars indicate 1 standard deviation above and below the mean. n.d. indicates no target mRNA was detected in the sample at the timepoint (note that in the IFN-α4 assay, only two data points for the Unf group are shown as only two of all of the samples tested possessed detectable levels of IFN-α4 mRNA). The mRNA level in uninfected spleens (Unf) is indicated by the dotted yellow line, with the shaded area defining 1 standard deviation above and below the maximum and minimum uninfected values.

detected in the spleen of LCMV Arm inoculated mice at 3 weeks post-infection, low but nonetheless slightly elevated levels of IFN-β and IFN-α4 mRNA were found to be present in the spleen of LCMV Cl13-infected mice.

4.3.2 Levels of Mx mRNA in the spleen during acute and persistent LCMV infections

The magnitude and kinetics of Mx mRNA upregulation following infection of mice with LCMV Arm and LCMV Cl13 were very similar. Mx 1 levels increased over the first 24 hours of infection, then began to decline, gradually returning to the levels observed in uninfected controls beyond day 7 post-infection (Figure 4.3D). In contrast to type 1 IFN mRNA levels, Mx mRNA levels in LCMV Cl13-infected mice were not found to be chronically elevated at 3 weeks post-infection.

4.4 Serum levels of type 1 IFN during LCMV Arm or LCMV Cl13 infection

Serum samples were collected in parallel with spleen tissue to enable measurement of systemic levels of type 1 IFN activity during acute and persistent LCMV infections. The L929-EMCV protection bioassay was employed to determine total bioactivity of type 1 IFN in the serum, with no distinction between IFN types or subtypes.

The kinetics of type 1 IFN production in mice infected with LCMV Arm or Cl13 were found to be very similar (Figure 4.4). In both groups of mice, type 1 IFN production was induced very rapidly after infection, with levels of activity in the serum reaching a peak within the first 48 hours of infection, well before peak in viral titres were reached (Figures 4.1 and 4.2). The peak serum IFN titre in LCMV Cl13-infected mice was slightly (~2-fold less) lower than the maximal level of circulating IFN recorded in

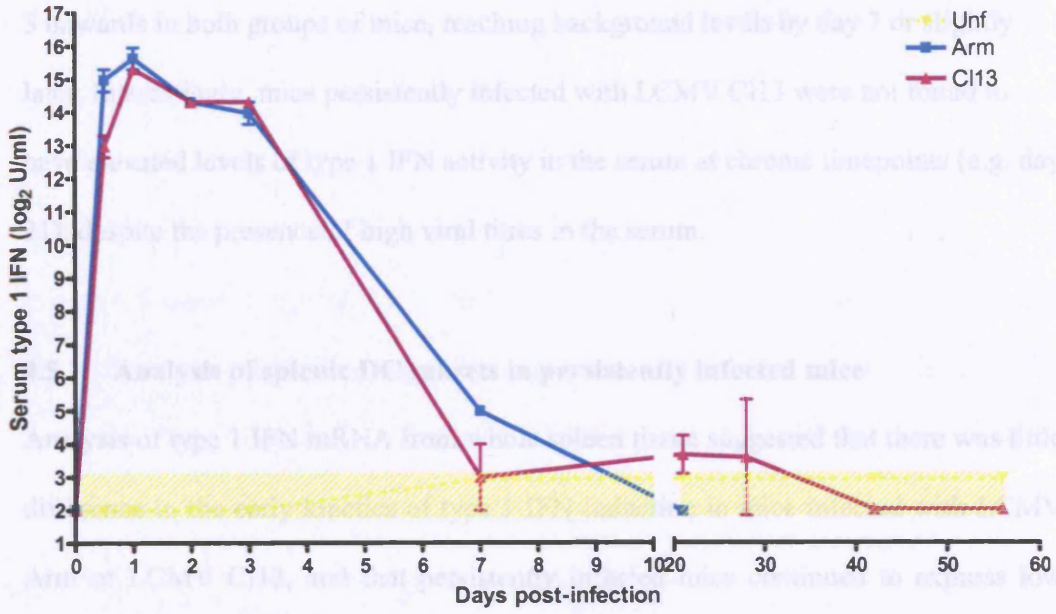

Figure 4.4 Type 1 IFN levels in the serum over time following infection of mice with LCMV Arm or Cl13

C57BL/6 mice were infected i.v. with $5x10^5$ p.f.u LCMV Arm or $5x10^6$ p.f.u LCMV Cl13. At the indicated times post-infection, blood samples were collected from groups of three mice infected with each virus or a single uninfected control mouse (Unf), and serum antiviral activity was determined by bioassay. The values shown are the mean serum IFN-α/β activity in groups of LCMV infected mice, with the error bars indicating 1 standard deviation above and below the mean. The maximal and minimal values of the uninfected (Unf) controls are indicated by the shaded area of the graph. The data shown are representative of results obtained from 3 independent experiments.

mice infected with LCMV Arm. Type 1 IFN levels in the serum fell rapidly from day 3 onwards in both groups of mice, reaching background levels by day 7 or slightly later. Interestingly, mice persistently infected with LCMV Cl13 were not found to have elevated levels of type 1 IFN activity in the serum at chronic timepoints (e.g. day 21), despite the presence of high viral titres in the serum.

4.5 Analysis of splenic DC subsets in persistently infected mice

Analysis of type 1 IFN mRNA from whole spleen tissue suggested that there was little difference in the early kinetics of type 1 IFN induction in mice infected with LCMV Arm or LCMV Cl13, and that persistently infected mice continued to express low levels of type 1 IFN mRNA throughout the course of the infection. Dendritic cells have been identified as important producers of type 1 IFNs during many virus infections, with pDCs being implicated as the source of much of the type 1 IFN found in serum in a number of infections (although this remains controversial for LCMV (Dalod et al. 2002)). Experiments were thus carried out to address changes taking place in the number and activation state of spleen DC subsets in LCMV-infected mice, and the role they may play in type 1 IFN production during acute and persistent LCMV infections.

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4.5.1 Identification of splenic DC subsets

To isolate splenic DCs, spleens pooled from a group of 6-10 mice were lysed, then low density cells were enriched on a Nycoprep gradient and DCs were isolated by positive selection on CD11c Macs columns. DC subsets were identified (and in some experiments isolated by Mo-Flo sorting) by staining with CD11c and B220 mAbs to distinguish DC subpopulations of the $CD11c^+$ $B220^+$ (plasmacytoid DC) or $CD11c^+$

$B220^-$ (myeloid or conventional DC) phenotype. In some experiments, plasmacytoid DCs within the $CD11c^+$ population were instead identified on the basis of pDCA-1 expression, which was a newly available marker reported to be specifically expressed by plasmacytoid DCs (Krug et al. 2004). Here, plasmacytoid DCs were defined as cells with $CD11c^+$ pDCA-1^+ surface marker expression.

Figure 4.5 shows a typical FACS profile of splenic DC subsets after enrichment and $CD11c^+$ column purification and staining with anti-CD11c-APC and anti-B220-FITC. The proportion of plasmacytoid DCs, defined by the $CD11c^{low}$ $B220^+$ phenotype, comprised ~10-20% of the total $CD11c^+$ population while the conventional DC population comprised ~60-80% of the total splenic DC population, as has been previously described (Asselin-Paturel et al. 2003). The percentage of $CD11c^+$ pDCA-1^+ cells is about 13% of the total $CD11c^+$ population which is similar to the value of the $CD11c^+$ $B220^+$ subpopulation.

CD11c, which is routinely used as a marker for DC is also expressed on some cell types other than DCs in the spleen of normal mice and is upregulated on many non-DC cell types when they become activated (Lin et al. 2003). To determine what proportion of $CD11c^+$ cells in the spleen of normal mice express markers identifying them as belonging to other cell lineages and how this may change in mice persistently infected with LCMV, $CD11c^+$ cells were isolated from spleens of uninfected and mice infected 3 weeks previously with LCMV Arm or LCMV Cl113 and were stained with anti-MHC-Class II FITC, anti-CD11c-APC plus one of a panel of markers specific for a range of different lymphocyte subsets: CD3 (for T-cells), CD19 (for B-cells), CD14 (for monocytes), DX5 (for NK cells), F4/80 (for macrophages) and pDCA-1 (for plasmacytoid DCs). Figure 4.6 shows the percentage of $CD11c^+$, MHC class II^{high} cells from each group of mice that also expressed the different lineage specific

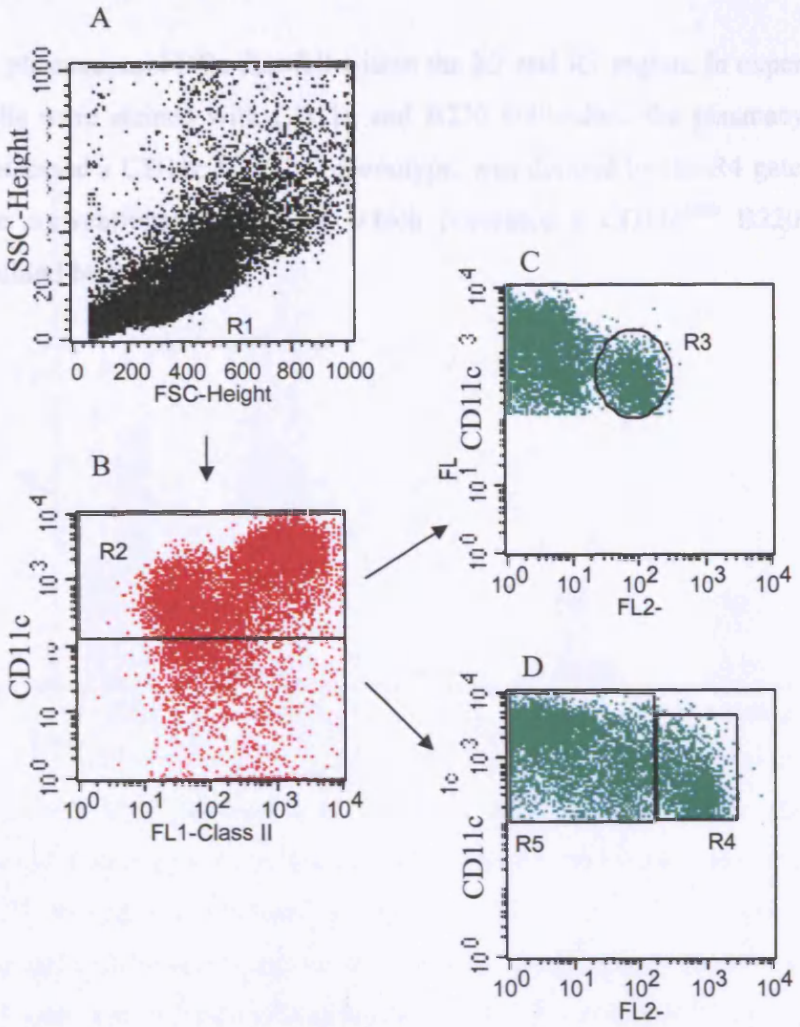

Figure 4.5 FACS plots of the gating scheme used to define the plasmacytoid and conventional DC subsets in isolated purified splenocytes

An example of the gating scheme used to identify the DC subsets is shown here using samples of lymphocytes isolated from pooled spleens from uninfected mice which were positively selected for CD11c expression. In some experiments, the isolated splenocytes were co-stained with mAbs to CD11c and pDCA-1 to identify the plasmacytoid DC population, while in other experiments, the splenocytes were co-stained with mAbs to CD11c and $B220^{+}$ to identify the plasmacytoid and conventional DC subsets on the basis of B220 expression. Dotplot A shows the forward and side scatter profile of the isolated splenocytes, with the R1 gate defining the live lymphocyte population. In dotplot B, R2 defines the $CD11c^{+}$ lymphocytes within the R1 region, and in dotplot C, R3 defines the population of $CD11c^{low}$ pDCA-

1^+ plasmacytoid DCs that fall within the R2 and R1 region. In experiments where the cells were stained with CD11c and B220 antibodies, the plasmacytoid DCs, which possessed a $CD11c^{low}$ $B220^+$ phenotype, was defined by the R4 gate in dotplot D and the conventional DC subset, which possessed a $CD11c^{high}$ $B220^-$ phenotype was defined by the R5 gate.

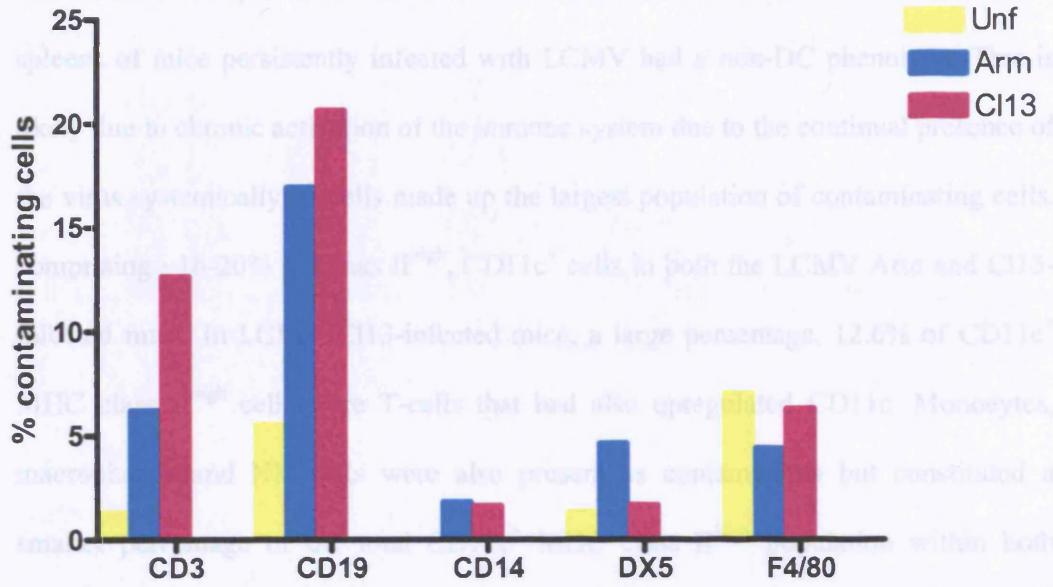

Figure 4.6 The percentage of CD11c$^+$ cells from uninfected animals and mice infected 3 weeks previously with LCMV which co-express non-DC markers

CD11c$^+$ lymphocytes isolated by positive selection from pooled spleens of uninfected mice and LCMV-infected mice at 3 weeks post-infection with LCMV Arm or LCMV Cl13 were stained with PE-conjugated mAbs to a range of lineage-specific markers, namely CD3 (T-cells), CD19 (B-cells), CD14 (monocytes), DX5 (NK cells) and F4/80 (macrophages). Cells were co-stained with CD11c-APC and MHC-class II-FITC. The % of CD11c$^+$, MHC-class II high cells expressing each non-DC marker is shown.

markers. There were more non-DC CD11c$^+$ MHC class II^{high} cells in cell populations derived from the spleens of LCMV-infected mice: almost 40% CD11c$^+$ cells from the spleens of mice persistently infected with LCMV had a non-DC phenotype. This is likely due to chronic activation of the immune system due to the continual presence of the virus systemically. B-cells made up the largest population of contaminating cells, comprising ~16-20% of Class II^{high}, CD11c$^+$ cells in both the LCMV Arm and Cl13-infected mice. In LCMV Cl13-infected mice, a large percentage, 12.6% of CD11c$^+$ MHC class II^{high} cells were T-cells that had also upregulated CD11c. Monocytes, macrophages and NK cells were also present as contaminants but constituted a smaller percentage of the total CD11c$^+$ MHC class II^{high} population within both LCMV Arm and Cl13-infected mice. In light of this observation, in subsequent experiments in which DCs were enriched from persistently-infected animals, PE-conjugated CD3, CD19, F4/80 and DX5 antibodies were routinely included in order to enable exclusion of these contaminating cell types from the DC populations being analysed or sorted.

4.5.2 Changes in the numbers of plasmacytoid DCs and conventional DCs in the spleen over time following infection of mice with LCMV

In order to investigate whether the kinetic changes in type 1 IFN mRNA/activity levels observed in the spleen and serum following infection of mice with LCMV were associated with alterations in DC subset composition, the total number of plasmacytoid DCs and conventional DCs present in the spleen was determined in a series of timepoints after LCMV Arm or LCMV Cl13 infection. CD11c$^+$ cells isolated from spleens pooled from infected and uninfected mice were stained with antibodies to CD11c, B220 and pDCA-1 to allow identification of the DC subsets as well as a

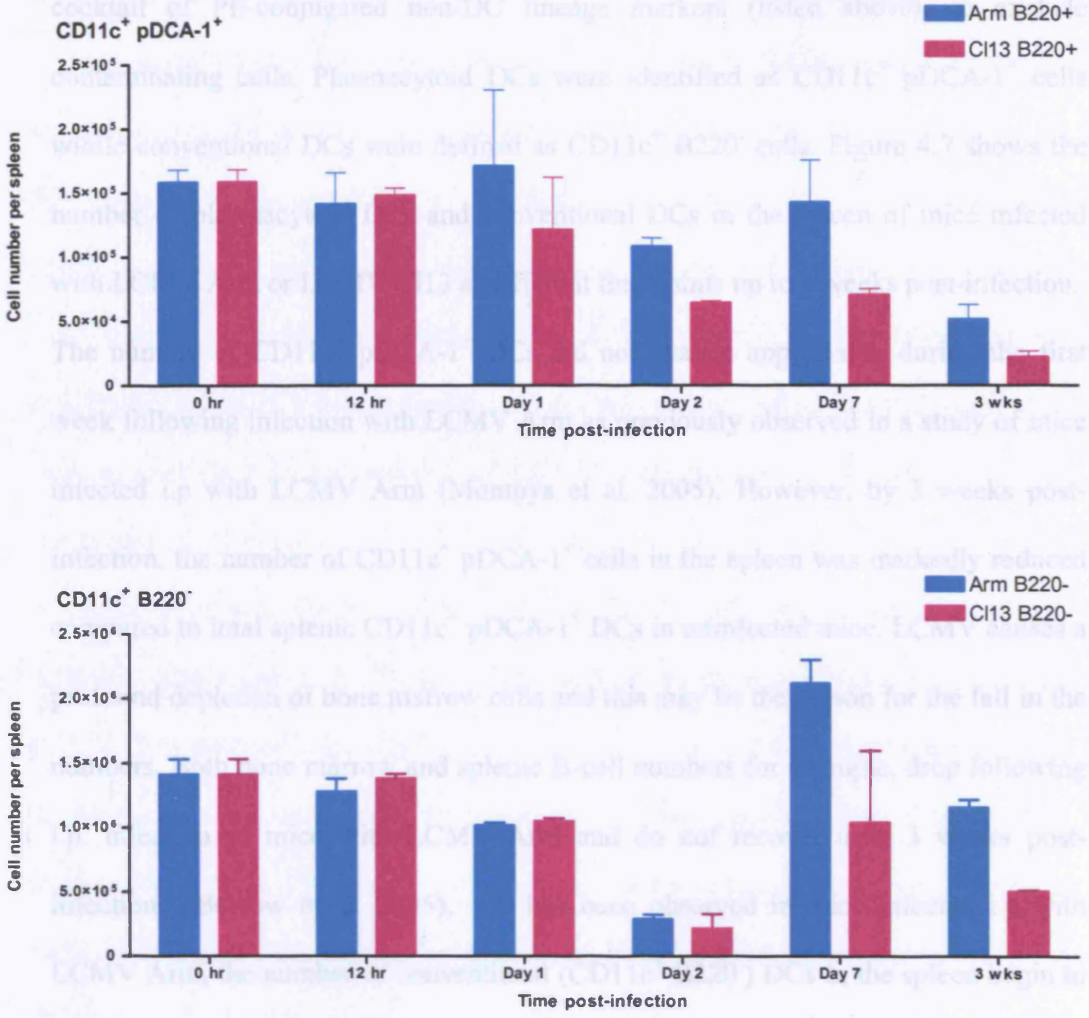

Figure 4.7 Numbers of CD11c$^+$ pDCA-1$^+$ and CD11c$^+$ B220$^-$ cells in the spleen during the course of an acute or persistent LCMV infection

CD11c$^+$ cells were isolated by positive selection from the spleens of groups of 10-20 mice infected with LCMV Arm or LCMV Cl13 at the indicated times post-infection. The isolated cells were stained with anti-CD11c-APC, B220-FITC or pDCA-1-PE and the total number of CD11c$^+$ pDCA-1$^+$ and CD11c$^+$ B220$^-$ cells per spleen was calculated The percentage of plasmacytoid DCs (B220$^+$, CD11c low) and conventional DCs (B220$^-$, CD11c high) in this fraction were determined by FACS analysis.. The values shown are the mean cell numbers obtained in 2-6 independent experiments, with the error bars indicating 1 standard error above the mean.

cocktail of PE-conjugated non-DC lineage markers (listed above), to exclude contaminating cells. Plasmacytoid DCs were identified as $CD11c^+$ $pDCA\text{-}1^+$ cells wihile conventional DCs were defined as $CD11c^+$ $B220^-$ cells. Figure 4.7 shows the number of plasmacytoid DCs and conventional DCs in the spleen of mice infected with LCMV Arm or LCMV Cl13 at different timepoints up to 3 weeks post-infection. The number of $CD11c^+$ $pDCA\text{-}1^+$ DCs did not change appreciably during the first week following infection with LCMV Arm as previously observed in a study of mice infected i.p with LCMV Arm (Montoya et al. 2005). However, by 3 weeks post-infection, the number of $CD11c^+$ $pDCA\text{-}1^+$ cells in the spleen was markedly reduced compared to total splenic $CD11c^+$ $pDCA\text{-}1^+$ DCs in uninfected mice. LCMV causes a profound depletion of bone marrow cells and this may be the reason for the fall in the numbers. Both bone marrow and splenic B-cell numbers for example, drop following i.p. infection of mice with LCMV Arm and do not recover until 3 weeks post-infection (Borrow et al. 2005). As has been observed in mice infected i.p with LCMV Arm, the number of conventional ($CD11c^+$ $B220^-$) DCs in the spleen begin to decline within a day of infection of mice with LCMV Arm and was markedly reduced compared to conventional DC numbers in uninfected mice by day 2 post-infection. The number of $CD11c^+$ $B220^-$ cells present in the spleen on day 7 post-infection was slightly higher than in uninfected animals. This coincides with the peak of the expansion of activated T-cells (Moskophidis et al. 1987) which may also have upregulated $CD11c^+$ as demonstrated in Figure 4.6. Anti-CD3 antibodies were added to the prep in order to exclude the contaminated T-cells, but it could be possible that the depletion was not complete due to the large numbers of activated T-cells at this timepoint (up to 43% of total splenocytes may be $CD11c^+$ $CD8^+$ T-cells (Lin et al. 2003)), and so there is a likelihood that a proportion of the $CD11c^+$ $B220^-$ cells in the

LCMV Arm samples are actually activated T-cells and not conventional DCs. By 3 weeks post-infection, the number of $CD11c^+$ $B220^-$ cells in the spleen had returned to normal. Again, the activated T-cell population may offer a possible explanation for this observation as expansion of the activated T-cell subset is followed by an apoptotic-mediated contraction phase that peaks about day 11 post-infection leading to a reduction in the T-cell numbers (Razvi and Welsh 1993). Thus the lower numbers of $CD11c^+$ $B220^-$ at 3 weeks post-infection may actually reflect the true number of conventional DCs in the spleens of LCMV Arm-infected mice during the later timepoints of infection.

A similar trend was observed with the $CD11c^+$ $B220^-$ DC subset after LCMV Cl13-infection. The number of $CD11c^+$ $B220^-$ DCs in the spleens of mice infected with LCMV Cl13 dropped markedly by day 2 post-infection, increased somewhat by day 7 post-infection and was again depressed at 3 weeks post-infection. One possible explanation for this observation may be that changes in the number of conventional DCs are being obscured by an increase in the number of non-DC contaminating the $CD11c^+$ $B220^-$ population on day 7 and 3 weeks post infection as may have occurred in the LCMV Arm –infected samples at similar timepoints. However, the number of contaminating activated T-cells may be lower than that in LCMV Arm –infected mice because of the abortive T-cell response induced in LCMV Cl13-infected mice, which results in lower numbers of activated $CD11c^+$ T-cells being present in the spleen.

The splenic CD11c $pDCA\text{-}1^+$ subset is less likely to be contaminated by non-DC cell types as they have been defined by a pDCA-1 marker specific for plasmacytoid DCs. The numbers of the $CD11c^+$ $pDCA\text{-}1^+$ population in the spleens of LCMV Arm mice did not change appreciably within the first 24 hours post-infection (Figure 4.7) and remained fairly stable over the first week post-infection, and were only depressed at 3

weeks post-infection. In contrast, there was a more rapid and profound decrease in the numbers of splenic plasmacytoid DCs of mice infected with LCMV Cl13, with the numbers being reduced relative to those in uninfected mice by day 2 post-infection, and remaining severely depressed at 3 weeks post-infection.

These results suggest that there were profound changes in the splenic DC numbers of mice following infection with LCMV Arm or LCMV Cl13. Notably, mice persistently infected with LCMV Cl13 experienced a sharp reduction in the numbers of plasmacytoid and conventional DCs present in the spleen. I would have liked to have conducted statistical analysis on the values (ANOVA General Linear Modal followed by Tukey's pairwise comparison) to determined whether the difference between the total splenic numbers in uninfected and LCMV-infected mice infected 3 weeks previously was significantly different; however, I did not posses enough experimental replicates to do so.

4.5.3 Analysis of the activation status of DC subsets in persistently infected mice

To enable the phenotype activation status of the low numbers of plasmacytoid DCs and conventional DCs that were present in the spleen of mice persistently infected with LCMV Cl13 to be addressed, $CD11c^+$ cells were enriched from the spleens of groups of uninfected mice and mice infected 3 weeks previously with LCMV Cl13, then a depletion step was carried out using biotinylated antibodies to appropriate lineage-specific markers to remove T cells, B cells and NK cells from the $CD11c^+$ population. Depleted cells were subsequently stained with streptavidin PE to determine whether all the contaminating subsets had been removed. Figure 4.8 shows the results obtained in a typical experiment. Here, undepleted and depleted $CD11c^+$

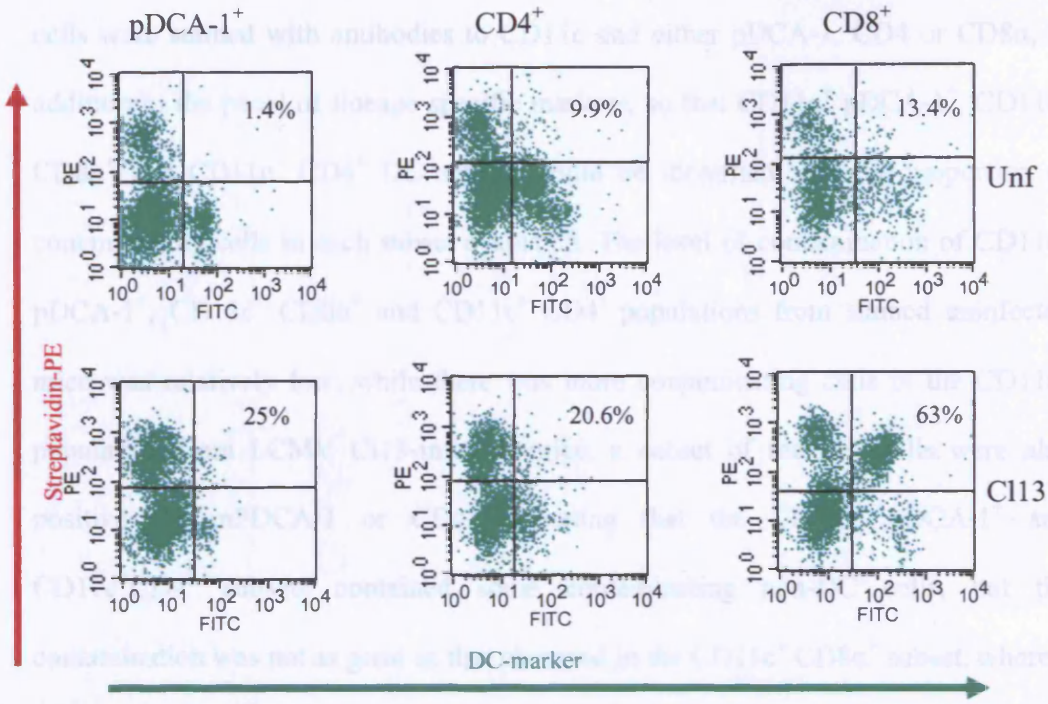

Figure 4.8 The percentage of CD11c$^+$ cells of different subsets from mice infected 3 weeks previously with LCMV Cl13 that co-express non-DC markers after negative selection

CD11c$^+$ lymphocytes isolated by positive selection from pooled spleens of uninfected (Unf) mice and mice infected 3 weeks previously with LCMV Cl13 were depleted of CD11c$^+$ cells expressing non-DC markers by staining with biotinylated mAbs to lineage-specific markers, CD3, CD19 and DX-5, followed by depletion using streptavidin conjugated Dynalbeads. The proportion of cells co-expressing non-DC lineage markers which remained after depletion was determined by FACS analysis. Aliquots of the purified cells were stained with streptavidin-PE and the mAbs CD11c-APC plus one of the following FITC-conjugated DC subset markers: pDCA-1, CD4 or CD8. The percentage of contaminating cells within each DC subset (CD11c$^+$, DC-marker$^+$, PE$^+$) cells, expressed as a percentage of all CD11c$^+$, DC-marker$^+$ cells is shown in the upper right quadrant of each dotplot. The results shown are representative of observations made in two independent experiments.

cells were stained with antibodies to CD11c and either pDCA-1, CD4 or CD8α, in addition to the panel of lineage specific markers, so that $CD11c^+$ $pDCA\text{-}1^+$, $CD11c^+$ $CD8\alpha^+$ and $CD11c^+$ $CD4^+$ DC subsets could be identified, and the proportion of contaminating cells in each subset evaluated. The level of contamination of $CD11c^+$ $pDCA\text{-}1^+$, $CD11c^+$ $CD8\alpha^+$ and $CD11c^+$ $CD4^+$ populations from stained uninfected mice was relatively low, while there was more contaminating cells in the $CD11c^+$ population from LCMV Cl13-infected mice, a subset of the PE^+ cells were also positive for mPDCA-1 or CD4, indicating that the $CD11c^+$ $pDCA\text{-}1^+$ and $CD11c^+CD4^+$ subsets contained some contaminating non-DC cells, but the contamination was not as great as that observed in the $CD11c^+$ $CD8\alpha^+$ subset, where a high proportion of PE-positive cells were present in the $CD11c^+$ $CD8\alpha^+$ subset which meant that this subset was heavily contaminated with non-DC cells (likely activated $CD8^+$ T-cells).

Experiments were then carried out in which $CD11c^+$ cells were isolated from the spleens of groups of uninfected or LCMV-Cl13-infected mice, depleted of non-DCs by the method described above, and co-stained with antibodies to CD11c and either CD4, CD8 or pDCA-1 together with antibodies to the following phenotypic markers: MHC class I, MHC class II, CD86, CD80, CD40 and ICAM-1. The mean fluorescence intensities of the expression of the activation markers on DCs of each subset in uninfected and infected mice were compared (Figure 4.9). Expression of most of the markers examined was upregulated on $CD11c^+$ $mpDCA\text{-}1^+$ and $CD11c^+$ $CD4^+$ DC subsets, from mice infected 3 weeks previously with LCMV Cl13, indicating these DCs were present in a heightened activation state. While $CD11c^+$ $pDCA\text{-}1^+$ DCs from infected mice had upregulated MHC and co-stimulatory markers, the level of activation marker expression on these cells was lower than that on the

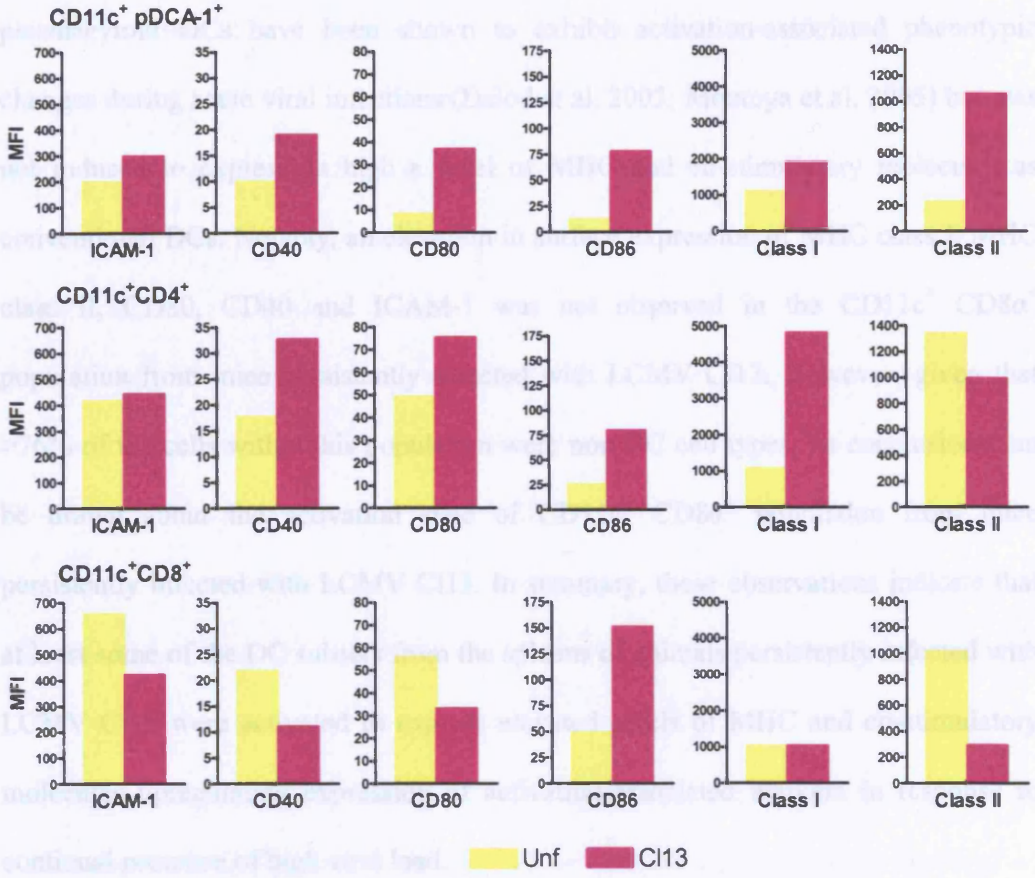

Figure 4.9 Phenotypic analysis of spleen DC subsets from mice persistently infected with LCMV Cl13

$CD11c^+$ cells were purified from the spleens of groups of 6-10 uninfected mice and mice infected 3 weeks previously with $5x10^6$ p.f.u. of LCMV Cl13. Purified cells were depleted of non-DC cells and then stained to allow identification of the indicated spleen DC subsets and co-stained with mAbs against adhesion, co-stimulatory and MHC molecules. The results shown here are the relative level of expression (mean fluorescence intensity (MFI)) of ICAM-1, CD40, CD80, CD86, MHC class I and MHC class II, as indicated, on each DC subset. The results shown are representative of findings made in two independent experiments.

$CD11c^+$ $CD4^+$ subset, in line with observations made in previous studies where plasmacytoid DCs have been shown to exhibit activation-associated phenotypic changes during acute viral infections (Dalod et al. 2003; Montoya et al. 2005) but was not induced to express as high a level of MHC and co-stimulatory molecules as conventional DCs. Notably, an elevation in surface expression of MHC class I, MHC class II, CD80, CD40 and ICAM-1 was not observed in the $CD11c^+$ $CD8\alpha^+$ population from mice persistently infected with LCMV Cl13. However, given that >76% of the cells within this population were non-DC cell types, no conclusions can be drawn about the activation state of $CD11c^+$ $CD8\alpha^+$ population from mice persistently infected with LCMV Cl13. In summary, these observations indicate that at least some of the DC subsets from the spleens of animals persistently infected with LCMV Cl13 were activated to express elevated levels of MHC and co-stimulatory molecules upregulating expression of activation-associated markers in response to continual presence of high viral load.

4.6 Characterization of type 1 IFN production by DC subsets during acute and persistent phases of LCMV infection

Previous studies from experiments carried out using total splenic tissue and serum showed that there was not high level of type 1 IFN production during persistent LCMV infection but nonetheless indicated that elevated levels of IFN-β and IFN-α4 were present in the spleen and serum of chronically infected animals. To gain insight into the roles of plasmacytoid DCs and conventional DCs in type 1 IFN production during the early and late phases of LCMV infection, a kinetic analysis of type 1 IFN mRNA levels in plasmacytoid DCs and conventional DCs isolated from the spleens of LCMV-infected mice was carried out. Pooled spleens from uninfected and mice

infected with LCMV Arm or LCMV Cl13 were lysed, then $CD11c^+$ cells were positively selected and sorted by Mo-Flo into two subsets, $CD11c^+$ $B220^+$ (plasmacytoid DC) or $CD11c^+$ $B220^-$ (conventional DC) populations. When samples from later timepoints (day 7 and 3 weeks) were processed, the $CD11c^+$ cells were co-stained with a cocktail of antibodies to T-cells, B-cells, macrophage and NK-cell markers so that cells of these lineages that had upregulated CD11c could be excluded from the sort. The number of cells of each sorted DC subset was determined and total RNA was extracted from an equal number of cells of each subset. The levels of IFN-β, IFN-α 4 and IFN-α (non-α 4) and Mx mRNA in each sample were then determined by quantitative real time Taqman RT-PCR. As shown in Figure 4.10 A, B, and C, type 1 IFN transcripts were strongly upregulated in the $CD11c^+$ $B220^+$ plasmacytoid DC subset within 12 hours of infection of mice with LCMV Arm or LCMV Cl13, and also some type 1 IFN mRNA upregulation was recorded in the $CD11c^+$ $B220^-$ conventional DC population, although not as marked an increase as occurred in plasmacytoid DCs. Type 1 IFN mRNA levels in plasmacytoid DCs were at their maximum at 12 hours post-infection, declining by day 1 post-infection and returning to baseline between days 2 and day 7 post-infection. The kinetics of type 1 IFN mRNA upregulation in the conventional DC population was somewhat slower, reaching their maximum on day 1 post-infection and then declining again between days 2 and 7 post-infection. There was no appreciable difference in type 1 IFN mRNA upregulation in plasmacytoid DCs/ conventional DCs in the acute phase of infection with LCMV Arm or LCMV Cl13. In mice infected with LCMV Arm, IFN-β and IFN-α4 mRNA levels in both plasmacytoid DCs and conventional DCs were below the limit of detection by 3 weeks post-infection, but during the persistent phase of infection, only the $CD11c^+$ $B220^+$ plasmacytoid DC subset continued to secrete a

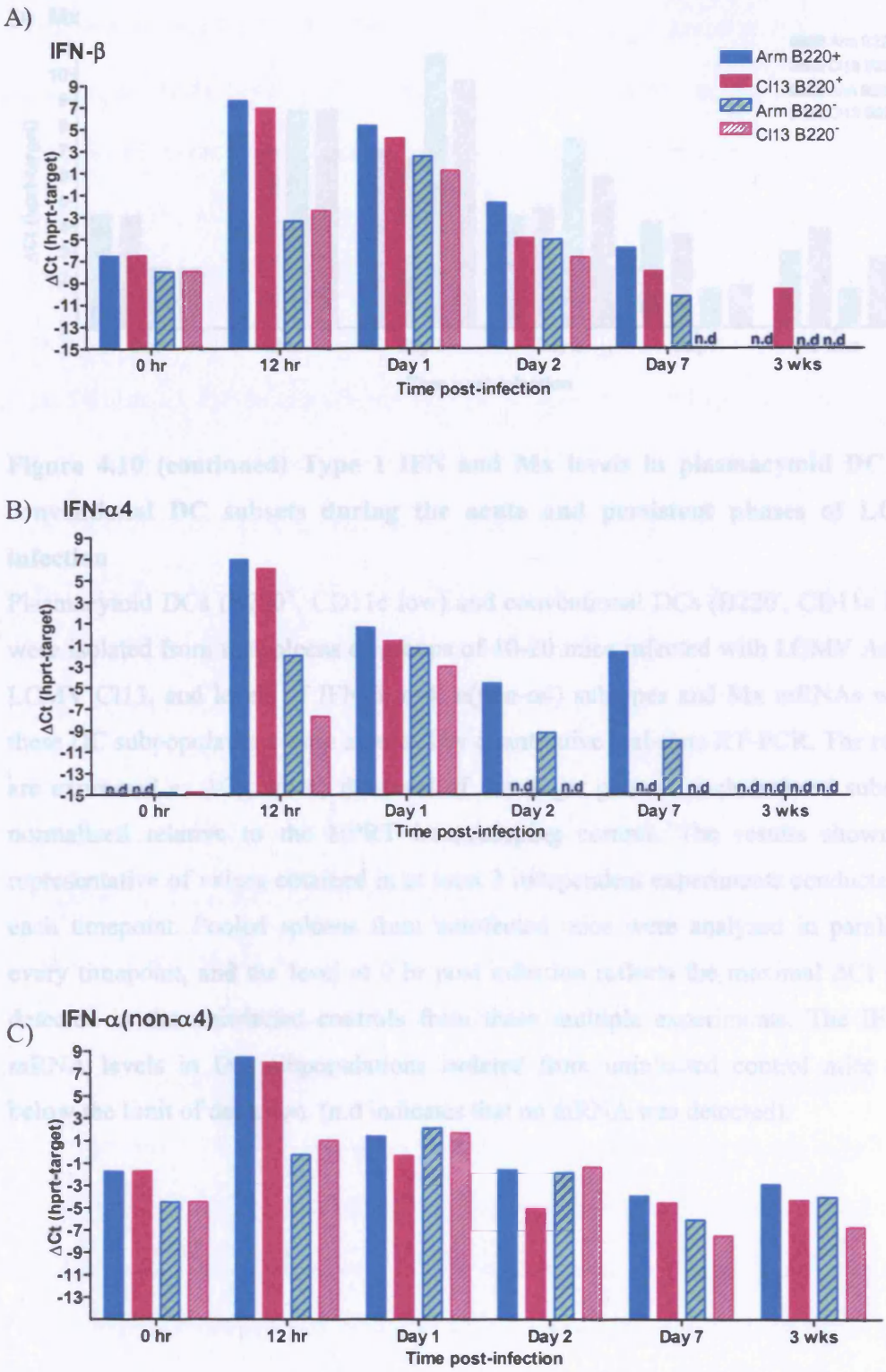

Figure 4.10 Type 1 IFN and Mx mRNA levels in plasmacytoid DC and conventional DC subsets during the acute and persistent phases of LCMV infection (figure continued on the following page)

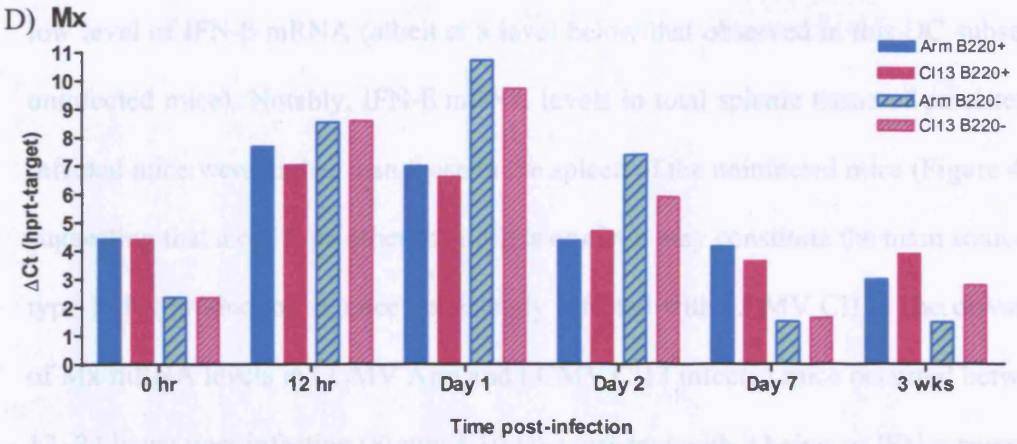

Figure 4.10 (continued) Type 1 IFN and Mx levels in plasmacytoid DC and conventional DC subsets during the acute and persistent phases of LCMV infection

Plasmacytoid DCs ($B220^+$, CD11c low) and conventional DCs ($B220^-$, CD11c high) were isolated from the spleens of groups of 10-20 mice infected with LCMV Arm or LCMV Cl13, and levels of IFN-β, α-4, α(non-α4) subtypes and Mx mRNAs within these DC subpopulations were assessed by quantitative real-time RT-PCR. The results are expressed as ΔCt, where the level of the target gene in each isolated subset is normalized relative to the HPRT housekeeping control. The results shown are representative of values obtained in at least 2 independent experiments conducted for each timepoint. Pooled spleens from uninfected mice were analysed in parallel at every timepoint, and the level at 0 hr post infection reflects the maximal ΔCt value detected in the uninfected controls from these multiple experiments. The IFN-α4 mRNA levels in DC subpopulations isolated from uninfected control mice were below the limit of detection. (n.d indicates that no mRNA was detected).

low level of IFN-β mRNA (albeit at a level below that observed in this DC subset in uninfected mice). Notably, IFN-β mRNA levels in total splenic tissue of persistently infected mice were higher than those in the spleen of the uninfected mice (Figure 4.3), suggesting that a cell type other than pDCs or cDCs may constitute the main source of type 1 IFN production in mice persistently infected with LCMV Cl13. The elevation of Mx mRNA levels in LCMV Arm and LCMV Cl13 infected mice occurred between 12- 24 hours post-infection (Figure 4.10 D) consistent with it being an IFN-responsive gene, the kinetics of upregulation of which would be expected to be delayed relative to those of type 1 IFN mRNA upregulation. As observed for type 1 IFN subtypes, maximal Mx expression occurred in the acute phase of infection with LCMV Arm or LCMV Cl13 and Mx mRNA levels returned to baseline by day 7 post-infection, remaining low during the persistent phase of LCMV infection.

4.6.1 Analysis of LCMV RNA levels in plasmacytoid and conventional DC populations from LCMV-infected mice

An important inducer of type 1 IFN production in cells is viral RNA; most cells are able to respond to the presence of viral RNA in the cytoplasm while DC subsets have the ability to detect the presence of viral nucleic acids in their external environment via expression of TLR 3/7 or 9. To gain insight into the levels of LCMV RNA associated with DC subsets (due to association of virion nucleic acids with these cells), real-time Taqman quantitative RT-PCR analysis was conducted on RNA extracted from plasmacytoid and conventional DC subsets isolated from the spleens of mice at different times following infection with LCMV Arm or LCMV Cl13, using primers that detected genome-sense LCMV NP RNA. As shown in Figure 4.11, similar levels of LCMV RNA were found to be associated with plasmacytoid DCs ($CD11c^+$ $B220^+$)

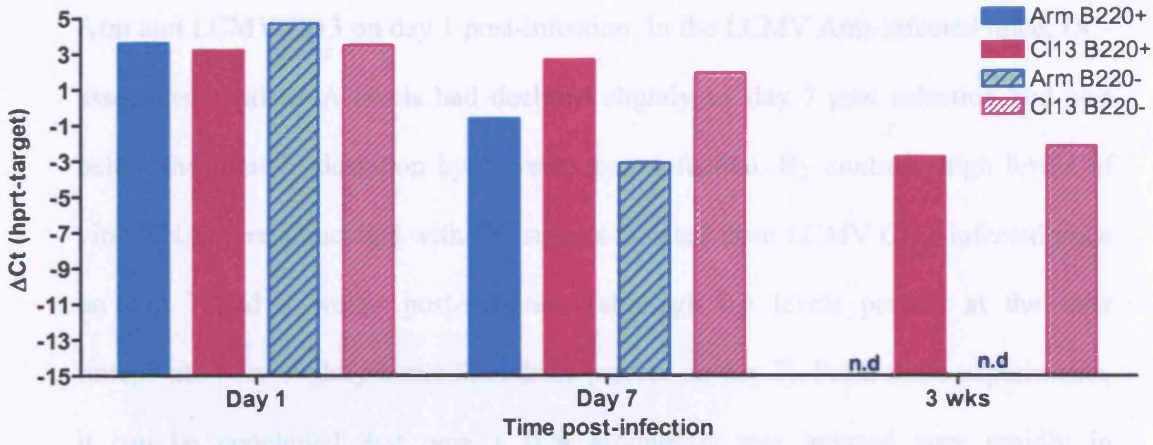

Figure 4.11 Levels of LCMV RNA in splenic plasmacytoid DC and conventional DC subsets of LCMV-infected mice during acute and chronic phases of LCMV infection

Plasmacytoid DCs ($B220^+$, CD11c low) and conventional DCs ($B220^-$, CD11c high) were isolated from the spleens of groups of 10-20 mice infected with LCMV Arm or LCMV Cl13 and the level of genome-sense LCMV NP RNA within these DC subpopulations was assessed by quantitative real-time RT-PCR. The results are expressed as ΔCt, where the level of the target gene in each isolated subset is normalized relative to the HPRT housekeeping control. The results shown are representative of values obtained in at least 2 independent experiments conducted for each timepoint. n.d indicates the level in the sample was below the limit of detection.

and conventional DCs ($CD11c^+$ $B220^-$) cell subsets from mice infected with LCMV Arm and LCMV Cl13 on day 1 post-infection. In the LCMV Arm-infected mice, DC-associated viral RNA levels had declined slightly by day 7 post infection and was below the limit of detection by 3 weeks post-infection. By contrast, high levels of viral RNA were associated with DC subsets isolated from LCMV Cl13-infected mice on day 7 and 3 weeks post-infection (although the levels present at the later timepoints were slightly lower than those present on day 7). From these experiments, it can be concluded that type 1 IFN production was induced very rapidly in plasmacytoid DCs, and with somewhat slower kinetics in conventional DCs following infection of mice with LCMV Arm or LCMV Cl13; however, high levels of type 1 IFN mRNA expression in these DC subsets was not sustained but was quickly downregulated, and remained low/undetectable in mice persistently infected with LCMV Cl13, despite constant exposure of these cells to viral LCMV RNA which was found to be associated with both DC subsets.

4.7 Confocal staining of spleen sections from acute and persistently infected mice

As stated above, type 1 IFN can be triggered in response to a cell becoming infected, or can be triggered by specialised cell subsets, including plasmacytoid DCs, that are able to detect the presence of pathogen components in their environment. To gain insight into whether type 1 IFN was predominantly produced by infected or uninfected cells in LCMV-infected mice, sections of spleen from mice infected with LCMV Arm or LCMV Cl13 were co-stained with antibodies to IFN-α and LCMV-NP. The sections were also co-stained with the plasmacytoid-specific antibody pDCA-1, to enable the level of infection of plasmacytoid DCs in LCMV-infected mice and the

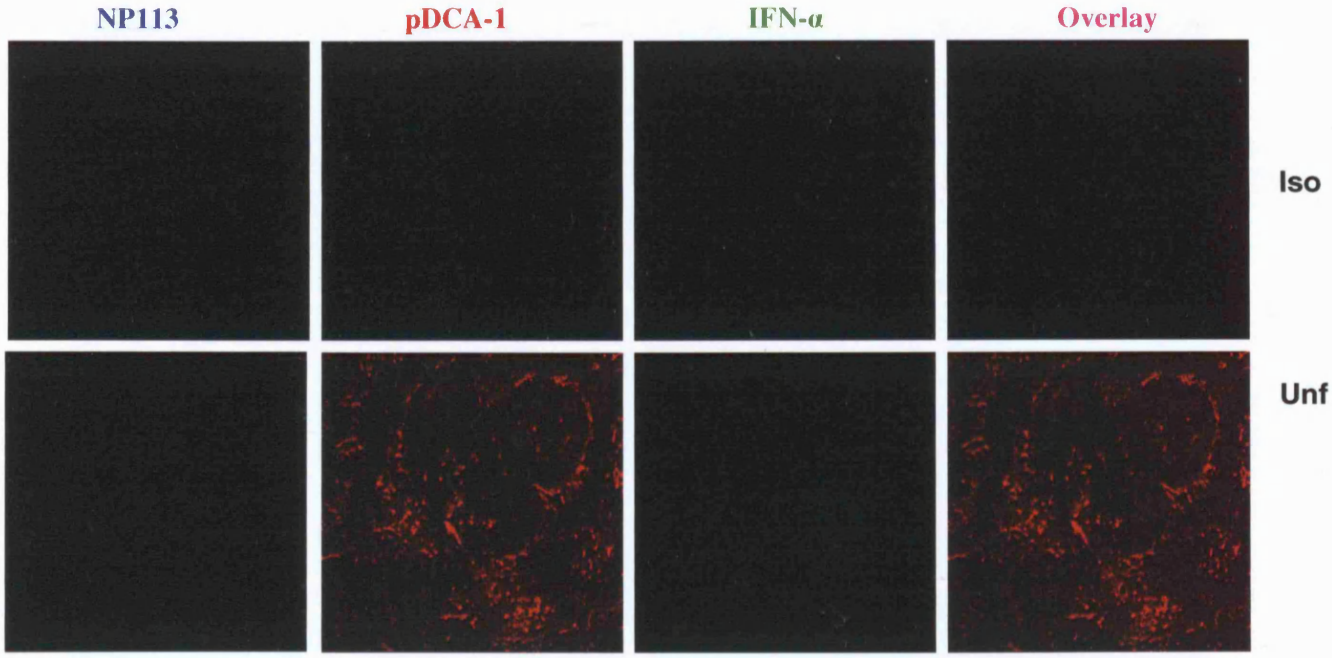

Figure 4.12 (continued) Immunofluorescent staining to identify IFN-α producing cells, LCMV-infected cells and plasmacytoid DCs in the spleens of LCMV-infected mice during the acute and persistent phases of infection

Cryosections from the spleens of uninfected mice and mice infected with LCMV Arm or Cl13 12 hours or 4 weeks previously were fixed and serially stained with the mAbs IFN-α-FITC, LCMV-NP-Cy5 and mpDCA-1-biotin followed by streptavidin-Tyr-Alexa 594 to enable identification of IFN-α producing cells (green), LCMV-infected cells (blue) and plasmacytoid DCs (red). Overlapping of IFN-α and plasmacytoid DCs staining produces a yellow overlay, IFN-α and LCMV-NP113 co-staining results in a pale blue colour, LCMV-NP113 and plasmacytoid DC co-localisation results in a

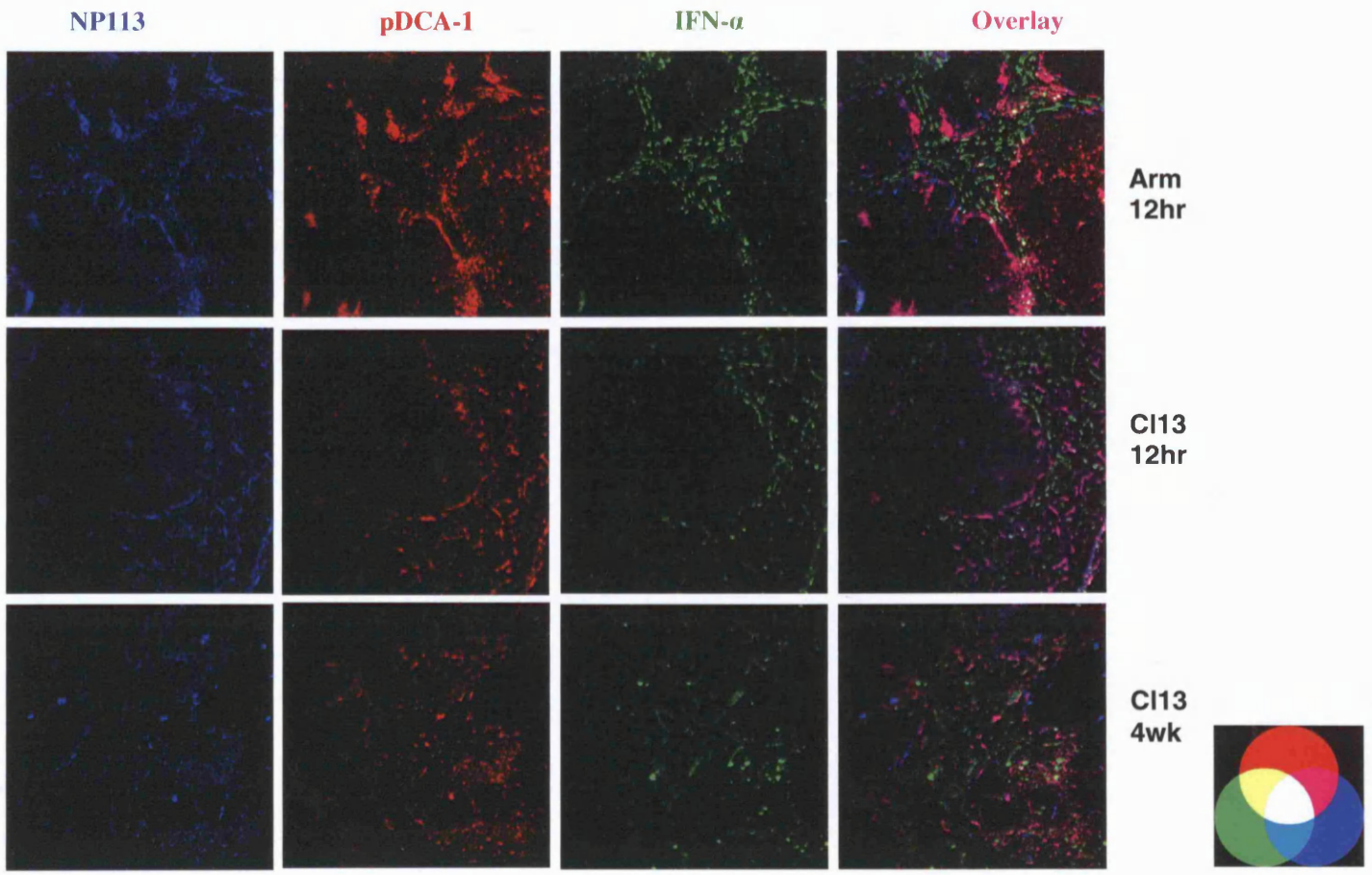

Figure 4.12 Immunofluorescent staining to identify IFN-α producing cells, LCMV-infected cells and plasmacytoid DCs in the spleens of LCMV-infected mice during the acute and persistent phases of infection (figure continued on the following page)

a white colour, as shown in the colour key. Appropriate isotype-matched control reagents were also tested. Images were captured at 10x magnification.

number of plasmacytoid producing IFN-α at different stages of the infection to be determined in parallel.

Spleens were excised, flash frozen and cryosectioned into 5μm sections and stained with IFN-α-FITC (green), LCMV-NP113-Cy5 (blue) and pDCA-1-biotin, followed by streptavidin-HRP and Tyr-594 substrate (red) to visualize IFN-α-producing, LCMV-infected and pDC cells in the spleen respectively. Staining of sections from uninfected mice revealed that plasmacytoid DCs were present in concentric patterns consistent with their reported location in the marginal zone (Figure 4.12). Staining of sections taken from the spleen of LCMV Arm and LCMV Cl13-infected mice at 12 hours post-infection indicated that pDCA-1 expression had been upregulated on the pDC surface by this time, possibly indicative of plasmacytoid DC activation. This was particularly evident in the mice infected with LCMV Arm but was also observed in LCMV Cl13-infected mice (although this is not obvious in the section shown in Figure 4.12). Further evidence of plasmacytoid DC activation at the timepoint in mice infected with both LCMV Arm and LCMV Cl13 is shown in Figure 4.13, which illustrates FACS analysis profiles of the forward and side scatter of isolated $CD11c^+$ $pDCA\text{-}1^+$ DCs from uninfected and infected mice: the cells from both groups of infected mice are enlarged and have increased in granularity. Many LCMV-infected cells were found to be present around the splenic marginal zones at 12 hours following infection of mice with LCMV Arm or LCMV Cl13. Notably a huge proportion of these appeared to be plasmacytoid DCs, as evidenced by co-localisation of NP113 and pDCA-1 staining in the overlay (magenta). IFN-α producing cells were also observed around the splenic marginal zones. Some of these were cell types other than plasmacytoid DCs which were not infected LCMV (green in the overlaid image) - but there was also evidence of type 1 IFN production by plasmacytoid DCs despite

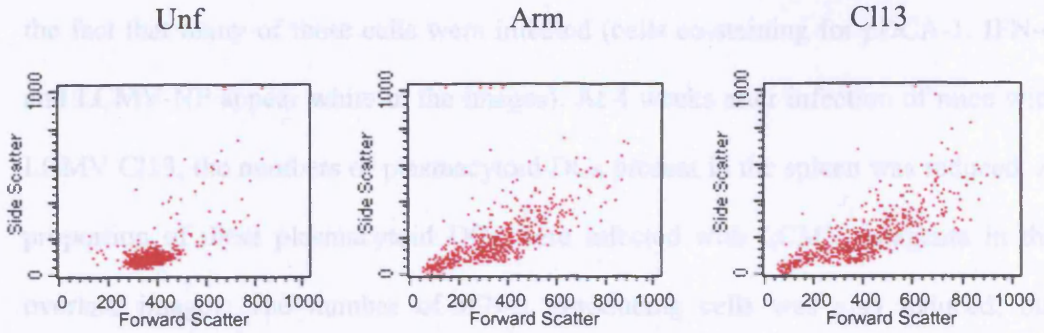

Figure 4.13 The morphology of CD11c$^+$ pDCA-1$^+$ cells from mice infected with LCMV

The forward and side scatter patterns of CD11c$^+$ pDCA-1$^+$ cells from uninfected or infected animals are shown. Groups of 6-10 mice were infected with LCMV Arm or LCMV Cl13 as previously described. At 24 hours post-infection, CD11c$^+$ pDCA-1$^+$ cells from the spleen were isolated, stained and analysed by FACS. CD11c$^+$ pDCA-1$^+$ cells were defined from the total cell population and the scatter profile of the gated cells were compared to determine whether infection with LCMV activates and alters the morphology of splenic CD11c$^+$ pDCA-1$^+$ cells. The results shown are representative of two experiments performed.

the fact that many of these cells were infected (cells co-staining for pDCA-1, IFN-α and LCMV-NP appear white in the images). At 4 weeks after infection of mice with LCMV Cl13, the numbers of plasmacytoid DCs present in the spleen was reduced. A proportion of these plasmacytoid DCs were infected with LCMV (magenta in the overlaid image). The number of IFN-α –producing cells was also reduced, but nonetheless some cells were producing IFN-α at this timepoint. Some of the IFN-α producing cells did not appear to be plasmacytoid DCs, but there were some which were plasmacytoid DCs. There was also very little co-localisation of IFN-α and NP113 staining, indicating that the majority of IFN-α producing cells were not infected with LCMV. However, a low number of LCMV Cl13-infected IFN-α producing pDCA-1^{+} plasmacytoid DC (white in the overlaid image) were observed in the spleen at this time.

There are some caveats to this analysis, firstly, NP is a virion structural protein, and hence the presence of NP may not necessarily be indicative of infection but may be the result of bound or endocytosed virions. However, given the widespread nature and intensity of cytoplasmic NP113 staining observed and the relatively small quantities of NP present within a virion, it is more likely that the NP113 staining seen in these experiments is reflective of LCMV infection of the NP positive cells. Secondly, NP expression may not have reached detectable levels in newly infected cells, hence it is possible that a higher proportion of plasmacytoid DCs and/or IFN-α producing cells may have been infected with virus than were detected in this experiment Thirdly, as the IFN-α monoclonal antibody used here only recognizes the IFN-α subtypes, these experiments do not provide any information on IFN-β production. Furthermore, because both the anti-IFN-α and the pDCA-1 antibodies were both rat monoclonals, the antibodies had to be applied in a step-wise manner, beginning with the anti-IFN-α

mAb and then the anti-rat-FITC, followed by the biotinylated anti-PDCA-1 and finally streptavidin-Tyr and the anti-NP-113 Cy5. Therefore, it can not be ruled out that steric hindrance from the anti-IFN-α or pDCA-1 antibodies may have prevented attachment of subsequent antibodies to the same cell. And finally, these experiments do not provide any information on the identity of the other IFN-α producing cells. Nonetheless, these experiments provide an insight into the cellular sources of type 1 IFN production in LCMV-infected mice, suggesting that in both the acute and chronic phases of infection, IFN-α is produced by uninfected cells that are not plasmacytoid DCs, and by a subset of plasmacytoid DCs, a subset of which appear to be infected.

4.8 Type 1 IFN production in persistently infected mice in response to exogenous stimulation with innate stimuli

Previous experiments showed that there is very little type 1 IFN expression in the spleen and serum of persistently infected mice, despite the presence of ongoing replication. To determine whether mice persistently infected with LCMV retained the capacity to respond to exogenous type 1 IFN-inducing stimuli, uninfected and LCMV Cl13-infected mice were inoculated i.p with ligands for TLR3 (polyIC) TLR7 (R848), TLR9 (CpG 1826 DNA) or with influenza PR8 which would activate TLR7 and/or the intracellular infection sensing pathway (Diebold et al. 2004; Prakash et al. 2005), and type 1 IFN and Mx mRNA levels in the spleen and type 1 IFN activity in the serum monitered at a series of timepoints thereafter.

4.8.1 PolyIC stimulation

In both uninfected mice and LCMV Cl13-infected mice, inoculation of the TLR3 ligand polyIC induced upregulation of all type 1 IFN mRNA subtypes in the spleen,

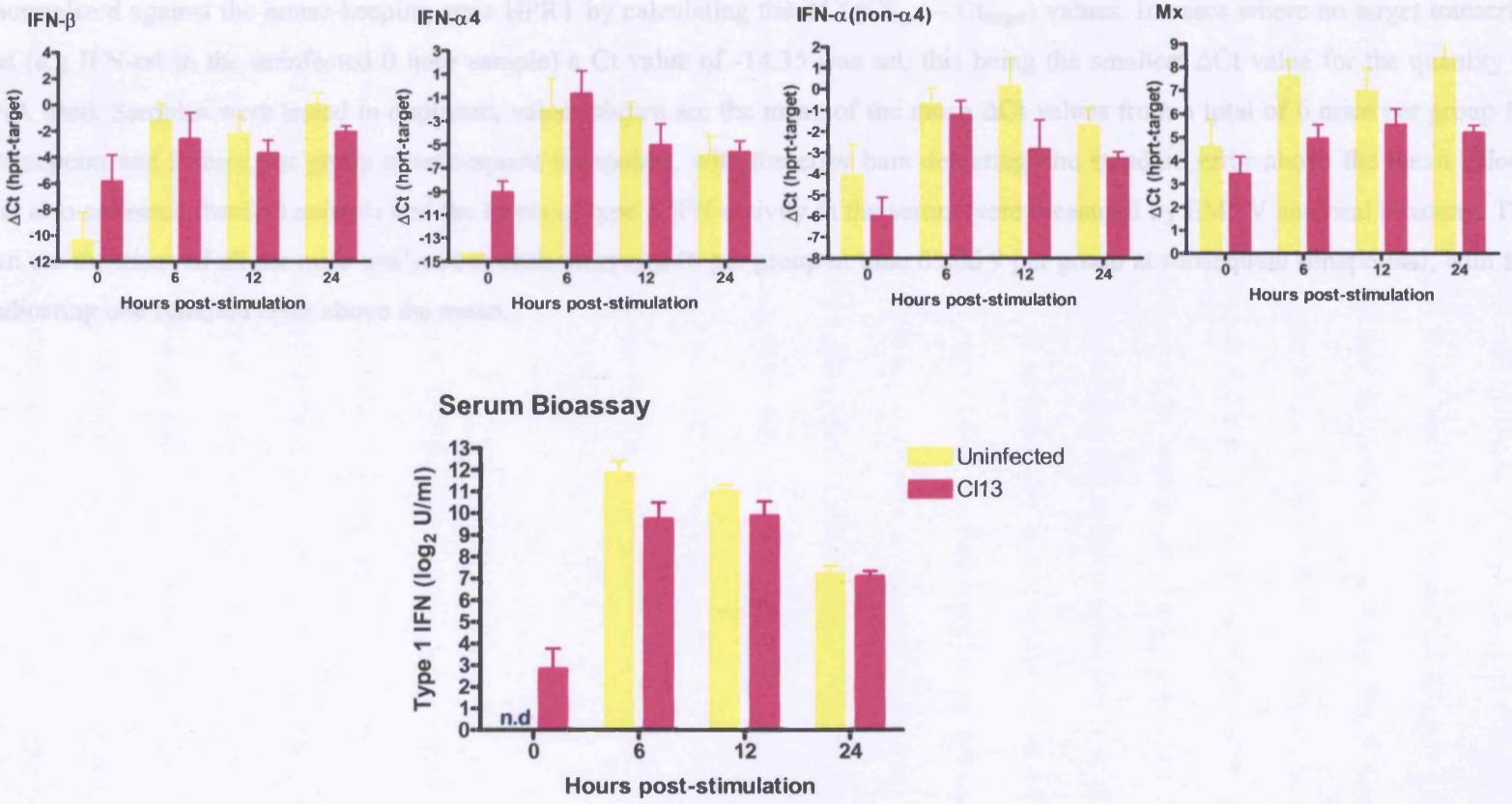

Figure 4.14 Type 1 IFN mRNA induction and serum type 1 IFN levels in persistently infected mice following inoculation with polyIC

IFN-β, IFN-α4, IFN-α (non-α4) subtype and Mx mRNA levels in the spleens of uninfected mice and mice persistently infected with LCMV Cl13 were assessed by real-time quantitative RT-PCR at the indicated timepoints after i.p. injection of 5µg of the TLR3 ligand polyIC. The results

have been normalized against the house-keeping gene HPRT by calculating the ΔCt ($Ct_{hprt} - Ct_{target}$) values. In cases where no target transcript was detected (e.g IFN-α4 in the uninfected 0 hour sample) a Ct value of -14.35 was set, this being the smallest ΔCt value for the quantity of template RNA used. Samples were tested in duplicate; values shown are the mean of the mean ΔCt values from a total of 6 mice per group for the 0 hour timepoint and 9 mice per group at subsequent timepoints, with the error bars depicting one standard error above the mean. Blood samples were also collected from all animals and the levels of type 1 IFN activity in the serum were measured by EMCV antiviral bioassay. The values shown are the mean of all the mice analysed at each timepoint (6 per group at time 0 and 9 per group at subsequent timepoints), with the error bars indicating one standard error above the mean.

with maximal type 1 IFN mRNA levels being reached by 6 hours post-infection and levels remaining elevated at 24 hours post-stimulation (Figure 4.14). The levels of bioactive type 1 IFN present in the serum mirrored this finding. There was no appreciable difference in the levels of type 1 IFN mRNA in the spleen or activity present in the blood of uninfected and LCMV Cl13-infected mice at 6, 12 or 24 hours post-stimulation. However, the uninfected mice upregulated type 1 IFN production to a greater extent than the infected animals as the baseline levels of IFN-β, -α4, -α (non-α4) transcripts were low or undetectable in the spleen of uninfected mice, while infected spleens contained pre-existing elevated levels of all the type 1 IFN-subtypes and low levels of type 1 IFN activity could be detected in the serum of LCMV Cl13-infected but not uninfected mice prior to inoculation of polyIC. Notably, Mx mRNA levels in the spleen of uninfected mice were found to be appreciably higher than those in infected mice at up to 24 hours post-stimulation possibly due to the greater increase in type 1 IFN production that occurred in the uninfected animals.

4.8.2 R848 stimulation

Inoculation of the TLR7 ligand R848 induced a very similar type 1 IFN expression in the spleen and blood of uninfected and infected mice (Figure 4.15). Notably, there was no appreciable difference in the levels of type 1 IFN mRNA present in the spleen or of type 1 IFN activity present in the serum of uninfected and infected mice at 6, 12 or 24 hours following R848 inoculation. However, as with polyIC, the extent of upregulation of type 1 IFN mRNA/activity was lower in infected than in uninfected mice, as the infected mice had pre-existing levels of type 1 IFN mRNA/activity. In association with this, Mx mRNA levels in the spleen of uninfected mice were again significantly higher than those in infected mice at 24 hours post-stimulation.

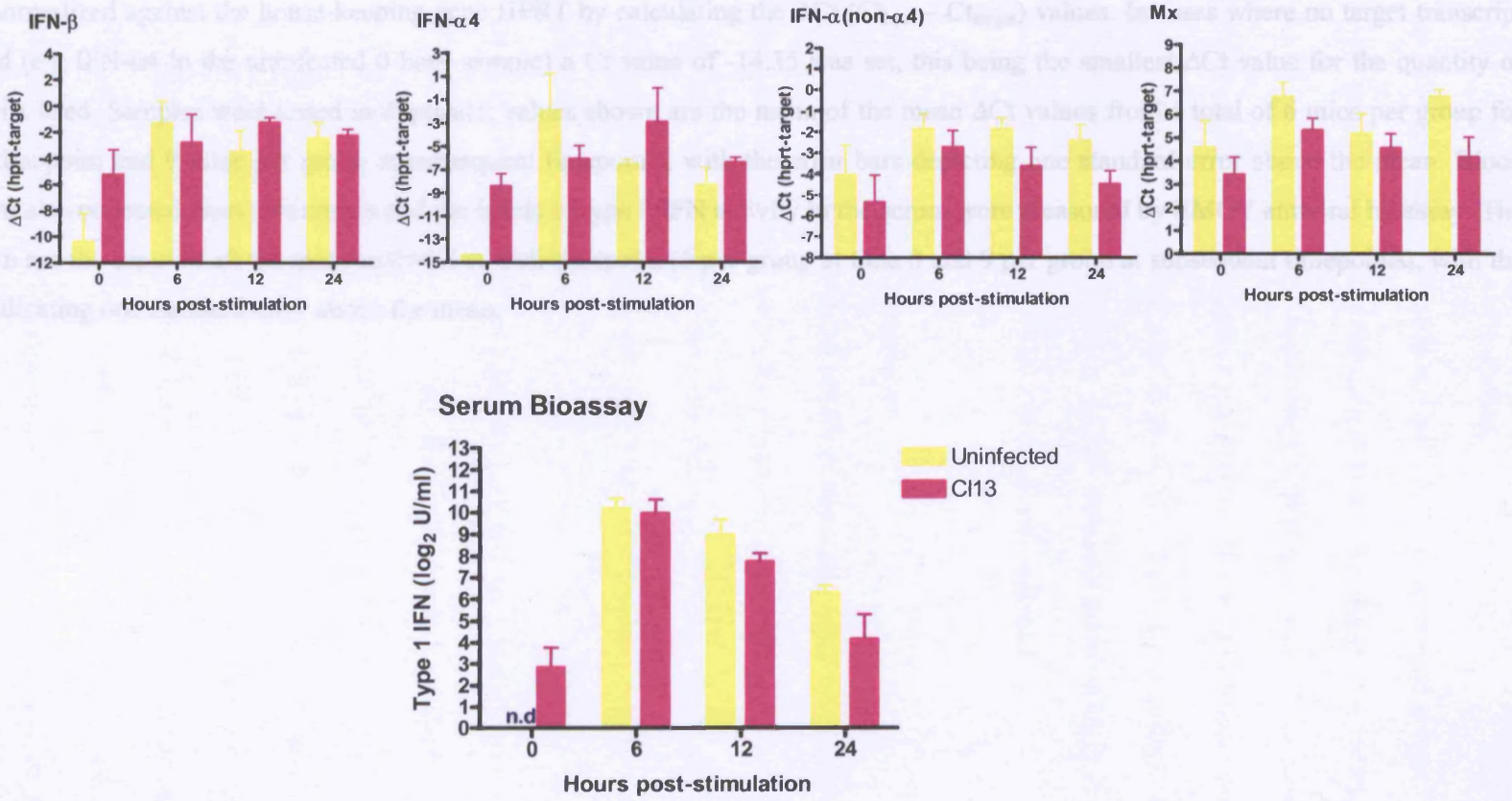

Figure 4.15 Type 1 IFN mRNA induction and serum type 1 IFN levels in persistently infected mice following inoculation with R848

IFN-β, IFN-α4, IFN-α (non-α4) subtype and Mx mRNA levels in the spleens of uninfected mice and mice persistently infected with LCMV Cl13 were assessed by real-time quantitative RT-PCR at the indicated timepoints after i.p injection of 25nmoles of the TLR7 ligand R848. The results

have been normalized against the house-keeping gene HPRT by calculating the ΔCt ($Ct_{hprt} - Ct_{target}$) values. In cases where no target transcript was detected (e.g IFN-α4 in the uninfected 0 hour sample) a Ct value of -14.35 was set, this being the smallest ΔCt value for the quantity of template RNA used. Samples were tested in duplicate; values shown are the mean of the mean ΔCt values from a total of 6 mice per group for the 0 hour timepoint and 9 mice per group at subsequent timepoints, with the error bars depicting one standard error above the mean. Blood samples were also collected from all animals and the levels of type 1 IFN activity in the serum were measured by EMCV antiviral bioassay. The values shown are the mean of all the mice analysed at each timepoint (6 per group at time 0 and 9 per group at subsequent timepoints), with the error bars indicating one standard error above the mean.

4.8.3 CpG 1826 DNA stimulation

Inoculation of the TLR9 ligand CpG 1826 DNA induced a type 1 IFN response in both uninfected and infected mice that was similar to that induced by polyIC and R848, but was of slightly lower magnitude (Figure 4.16). Once again, however, there was no significant difference in spleen type 1 IFN mRNA levels or serum IFN activity in infected and uninfected mice at the 6, 12 or 24 hour timepoints, although the extent of upregulation was smaller in LCMV-infected mice which had already low pre-existing levels of type 1 IFN than in uninfected animals.

4.8.3 Influenza PR8 stimulation

Intraperitoneal inoculation of influenza virus into mice introduces the virus into the peritoneal cavity, from which some systemic spread of virions occur. When influenza virus PR8 is introduced to mice via this route, viral entry into cells and a single round of replication can occur, but not subsequent spread of viral infection as virion infectivity is

dependent on the activity of host proteases that are not expressed systemically (Kido et al. 1999). The presence of infection could be detected via a range of receptors, including TLRs and intracellular pathways for stimulation of type 1 IFN production. Uninfected mice generated a large type 1 IFN response following inoculation of influenza virus (Figure 4.17). Peak type 1 IFN mRNA levels in the spleen and activity levels in the blood which were reached at 6-12 hours post-infection (slightly later than following inoculation of synthetic TLR ligands), were higher than those reached in mice inculated with TLR ligands and levels still remained high at 24 hours post-inculation. Notably however, there was only a slight upregulation of type 1 IFN mRNA expression in the spleen of LCMV Cl13-infected mice following inoculation

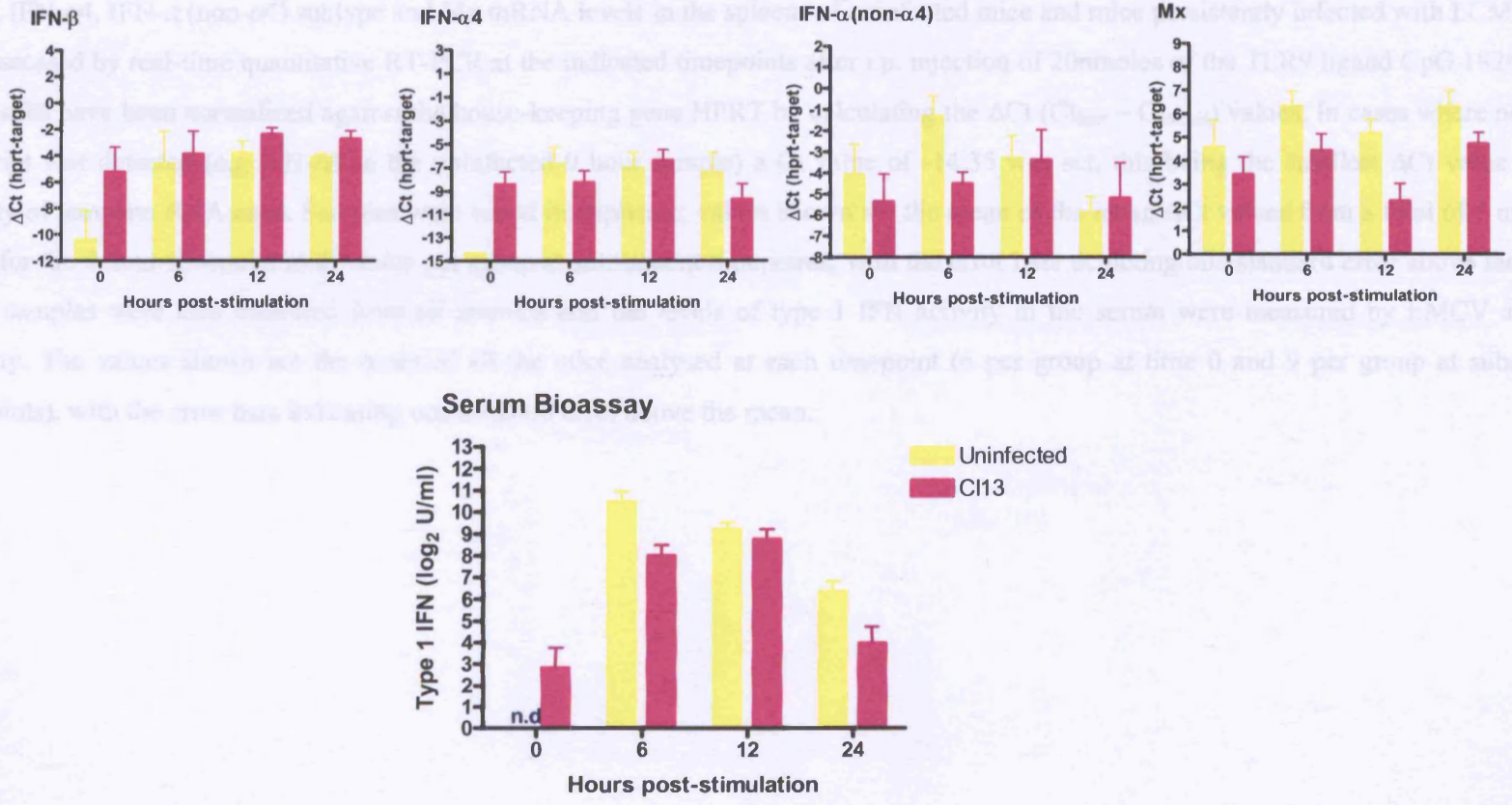

Figure 4.16 Type 1 IFN mRNA induction and serum type 1 IFN levels in persistently infected mice following inoculation with CpG 1826 DNA (legend on the following page)

IFN-β, IFN-α4, IFN-α (non-α4) subtype and Mx mRNA levels in the spleens of uninfected mice and mice persistently infected with LCMV Cl13 were assessed by real-time quantitative RT-PCR at the indicated timepoints after i.p. injection of 20nmoles of the TLR9 ligand CpG 1826 DNA. The results have been normalized against the house-keeping gene HPRT by calculating the ΔCt ($Ct_{hprt} - Ct_{target}$) values. In cases where no target transcript was detected (e.g IFN-α4 in the uninfected 0 hour sample) a Ct value of -14.35 was set, this being the smallest ΔCt value for the quantity of template RNA used. Samples were tested in duplicate; values shown are the mean of the mean ΔCt values from a total of 6 mice per group for the 0 hour timepoint and 9 mice per group at subsequent timepoints, with the error bars depicting one standard error above the mean. Blood samples were also collected from all animals and the levels of type 1 IFN activity in the serum were measured by EMCV antiviral bioassay. The values shown are the mean of all the mice analysed at each timepoint (6 per group at time 0 and 9 per group at subsequent timepoints), with the error bars indicating one standard error above the mean.

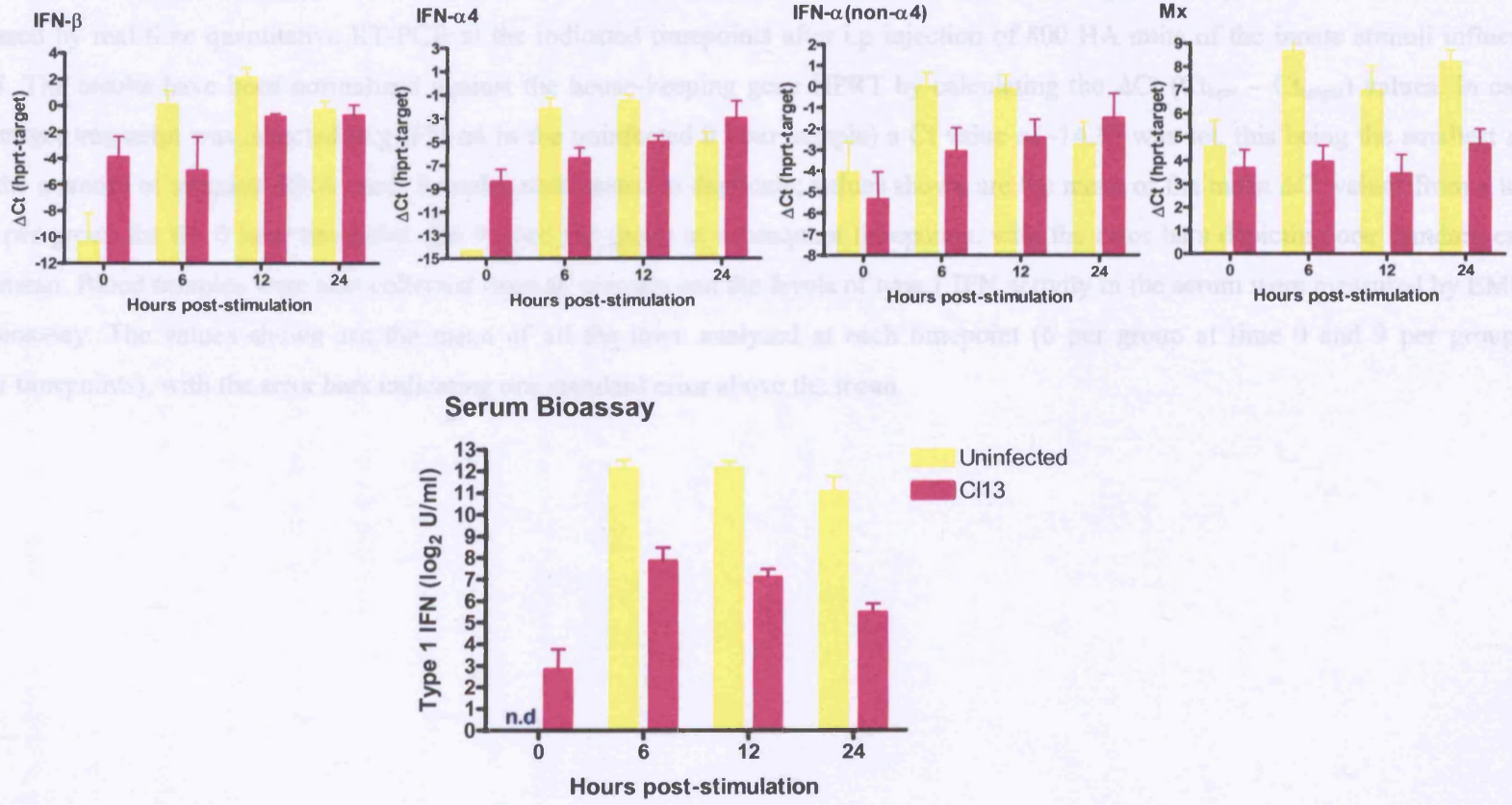

Figure 4.17 Type 1 IFN mRNA induction and serum type 1 IFN levels in persistently infected mice following inoculation with influenza virus strain PR8 (legend on the following page)

IFN-β, IFN-α4, IFN-α (non-α4) subtype and Mx mRNA levels in the spleens of uninfected mice and mice persistently infected with LCMV Cl13 were assessed by real-time quantitative RT-PCR at the indicated timepoints after i.p injection of 800 HA units of the innate stimuli influenza strain PR8. The results have been normalized against the house-keeping gene HPRT by calculating the ΔCt ($Ct_{hprt} - Ct_{target}$) values. In cases where no target transcript was detected (e.g IFN-α4 in the uninfected 0 hour sample) a Ct value of -14.35 was set, this being the smallest ΔCt value for the quantity of template RNA used. Samples were tested in duplicate; values shown are the mean of the mean ΔCt values from a total of 6 mice per group for the 0 hour timepoint and 9 mice per group at subsequent timepoints, with the error bars depicting one standard error above the mean. Blood samples were also collected from all animals and the levels of type 1 IFN activity in the serum were measured by EMCV antiviral bioassay. The values shown are the mean of all the mice analysed at each timepoint (6 per group at time 0 and 9 per group at subsequent timepoints), with the error bars indicating one standard error above the mean

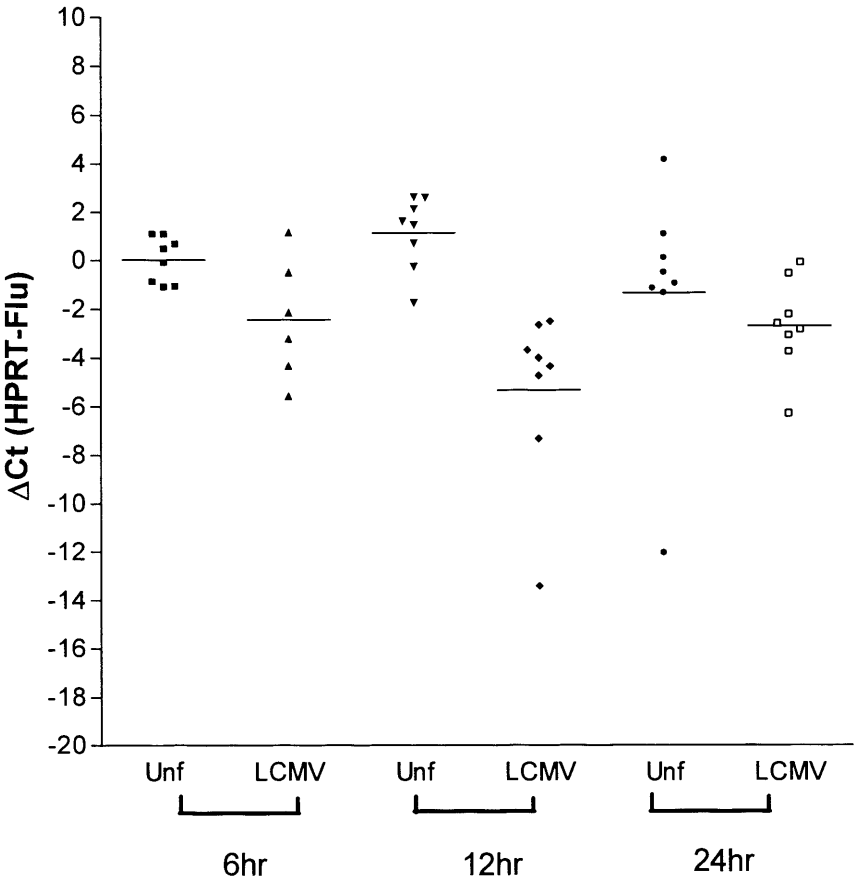

Figure 4.18 Levels of influenza virus RNA in spleens of uninfected and LCMV Cl13-infected mice following inoculation with influenza strain PR8

Uninfected (Unf) and LCMV Cl13-infected mice (at 3 weeks post-infection) were injected i.p. with 800 HA units of influenza virus strain PR8. At the indicated timepoints, 3 mice per group were sacrificed and their spleens removed for analysis. The level of influenza virus RNA that was in the spleen was determined by quantitative real-time RT-PCR using primers specific for the influenza A RNA genome. The results are expressed as ΔCt, i.e, the level of influenza virus RNA has been normalized relative to the housekeeping gene HPRT. Each point on the chart represents the influenza ΔCt value of a single mouse. The results from 3 independent experiments using 3 mice per group per timepoint are depicted. No influenza virus

RNA was detected in the spleens of uninfected or Cl13-mice that were not inoculated with influenza virus.

with influenza virus and serum levels of type 1 IFN activity in these mice were appreciably lower than those in uninfected mice at all post-inculation timepoints. Mx levels in LCMV Cl13 infected mice also remained unchanged over time, in contrast to the strong Mx upregulation in uninfected controls. Influenza viral RNA was detected from total RNA preps of the spleen by quantitative Taqman qRT-PCR. Higher levels of influenza PR8 transcripts were present in the total RNA of uninfected spleen when compared with the levels detected in LCMV Cl13 infected splenic cells.

Persistently infected mice may have a pre-existing anti-viral state that makes them resistant to subsequent viral infection. To investigate whether the poor type 1 IFN induction observed following i.p. inoculation of influenza PR8 into LCMV Cl13-infected mice was associated with failure of influenza virus to infect the spleen of these animals, the levels of influenza viral RNA in the spleen of uninfected and LCMV Cl13-infected animals were determined at different times post-infection. Analysis by real time quantitative RT-PCR indicated that influenza PR8 genomic material could be detected in the spleen of LCMV Cl13-infected mice (Figure 4.18), although the levels present were lower than those observed in uninfected mice at all timepoints tested. The PCR primers here would have detected both input influenza virus RNA and genomic-sense RNA generated in infected cells. The relatively low influenza virus RNA levels observed in the spleen of LCMV Cl13-infected mice may thus have reflected reduced virion trapping in the spleen and/or impaired viral replication in the spleen of these animals. This likely contributed to the low type 1 IFN response in these animals.

4.9 The level of inflammatory cytokines in persistently infected mice after stimulation with innate stimuli

Viral infection and innate stimuli induce production of many other cytokines in addition to type 1 IFNs. To gain insight into the effect of a persistent LCMV infection on the levels and pattern of production of other cytokines elicited following i.p. inoculation of polyIC, CpG 1826 DNA, R848 or influenza PR8, blood collected from uninfected and persistently infected mice at 6 hours post-stimulation and serum levels of the inflammatory cytokines IL-12p70, TNF-α and IFN-γ as well as IL-10, IL-6 and the chemotactic factor MCP-1 were measured by CBA assay. Initial experiments measured the levels at 6, 12 and 24 hours post-stimulation, but the 6 hour timepoint was selected as it was the timepoint with the highest level of upregulation.

The results obtained are shown in Figure 4.19. Prior to stimulation, only low or undetectable amounts of all the factors were present in the serum of both uninfected and LCMV Cl13-infected mice. Administration of polyIC, R848 and influenza to uninfected mice induced very little or no production of IL-12, IFN-γ or TNF-α at 6 hours post-inoculation, although a moderate elevation in serum levels of all 3 cytokines was observed following i.p. inoculation of CpG1826 DNA. Notably mice that were persistently infected with LCMV produced lower amounts of bioactive IL-12p70 and IFN-γ upon CpG1826 DNA inoculation than their uninfected counterparts; but they produced higher levels of TNF-α. Administration of polyIC and R848 into persistently infected mice also induced an upregulation of TNF-α levels in the serum, which was not observed in uninfected animals inoculated with these TLR ligands.

TLR ligands also elicited production of higher levels of IL-10 and IL-6 in LCMV-infected mice than were induced in uninfected animals.

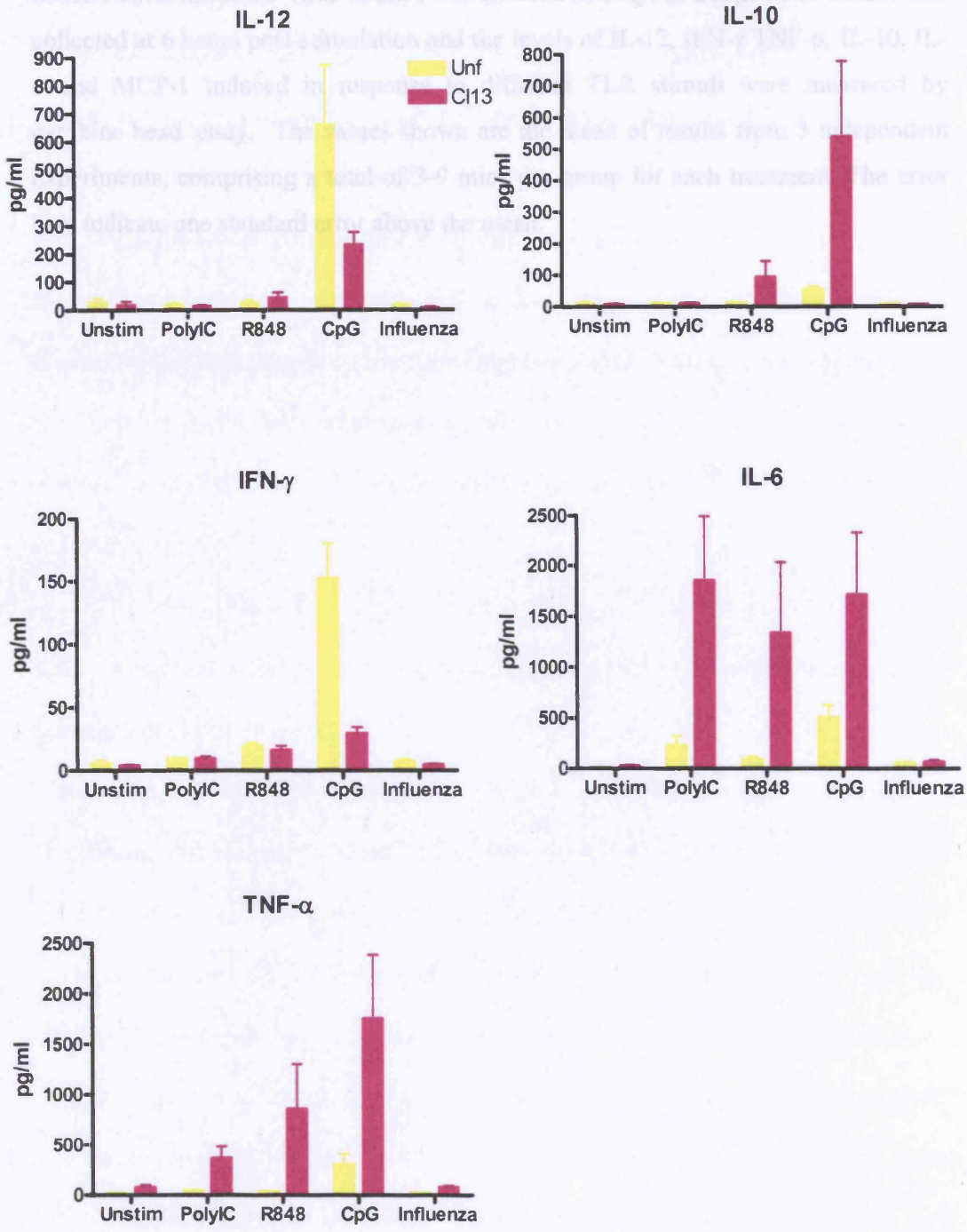

Figure 4.19 Levels of inflammatory cytokines induced in uninfected mice and mice persistently infected with LCMV Cl13 in response to TLR stimuli or influenza virus

Uninfected mice and mice persistently infected with LCMV Cl13 (at 3 weeks post-infection) were inoculated with 5μg polyIC, 20nmoles CpG 1826, 25nmoles R848 or

800HA units influenza virus strain PR8, all delivered i.p. in 200µl PBS. Serum was collected at 6 hours post-stimulation and the levels of IL-12, IFN-γ TNF-α, IL-10, IL-6 and MCP-1 induced in response to different TLR stimuli were measured by cytokine bead assay. The values shown are the mean of results from 3 independent experiments, comprising a total of 3-9 mice per group for each treatment. The error bars indicate one standard error above the mean.

PolyIC, R848 and CpG DNA all stimulated an increase in serum IL-6 levels in uninfected animals, and CpG DNA and to a lesser extent R848 also elicited production of very low levels of IL-10. These responses were all enhanced in LCMV Cl13-infected mice. I.p injection of influenza PR8 did not induce upregulation of IL6 or IL-10 in the serum of either groups of mice at the 6 hour timepoint.

In summary, there were qualitative differences in the pattern of cytokine production elicited by the TLR ligands in uninfected and persistently infected mice. Interestingly, the cytokine profile of persistently infected mice appeared to be skewed towards expression of TNF-α, IL-6 and IL-10 while production of IL-12 and IFN-γ was reduced.

4.10 Confocal staining of spleen sections of persistently infected mice after inoculation of innate stimuli

It was unclear which cells in the spleen were secreting type 1 IFN in response to stimulation with polyIC, CpG 1826 DNA, R848 or influenza PR8. In uninfected mice, splenic pDCs are reported to express high levels of TLR7 and TLR9 and produce large amounts of type 1 IFN in response to these stimuli, while other DC subsets, principally the $CD8\alpha^+$ DC subset are better adapted to recognize and respond to TLR3 ligands (Schulz et al. 2005). To investigate the role of plasmacytoid DCs versus other cell types in type 1 IFN production in response to different TLR stimuli in mice persistently infected with LCMV and to determine the contribution made by LCMV infected and uninfected cells to type 1 IFN production, uninfected and mice persistently infected mice inoculated for 6 hours previously with innate stimuli were subjected to immunohistochemical analysis as described in section 2.7.7.2 to visualize

pDCA-1 plasmacytoid DCs (red), IFN-α expressing cells (green) and LCMV-infected cells (blue). Figure 4.20 shows images in which all three colours have been overlaid.

In uninfected mice, multiple IFN-α producing cells were observed in the spleen of animals given each of the innate stimuli. In CpG, R848 and influenza-inoculated animals, many (although not all) of the IFN-α producing cells appeared to be plasmacytoid DCs (yellow or bright green). Not all plasmacytoid DCs were producing IFN-α at this timepoint. By contrast, the majority of IFN-α producing cells in polyIC-stimulated mice were not plasmacytoid DCs, but were cells around the outside of the marginal zone. These observations are consistent with published reports that plasmacytoid DCs express high levels of TLR7 (a receptor for R848 and influenza viral RNA) and TLR9 (a ligand for CpG), whilst other DC subsets, including $CD8\alpha^+$ DCs, which are located around the marginal zone in the resting spleen express high levels of TLR3 (which recognize polyIC) (Edwards et al. 2003).

As observed in previous experiments (Figure 4.12), reduced numbers of plasmacytoid DCs were present in the spleen of mice persistently infected with LCMV Cl13. A proportion of these plasmacytoid DCs were infected with LCMV Cl13 (magenta), but not all infected cells were plasmacytoid DCs. Inoculation of polyIC, R848, CpG and influenza virus into LCMV Cl13-infected mice resulted in an increase in the number of IFN-α producing cells in the spleen, although the increase was less marked than that observed in uninfected mice, consistent with the results of type 1 IFN mRNA analysis in Figures 4.14 – 4.17. As in uninfected mice, the majority of cells induced to produce IFN-α following inoculation

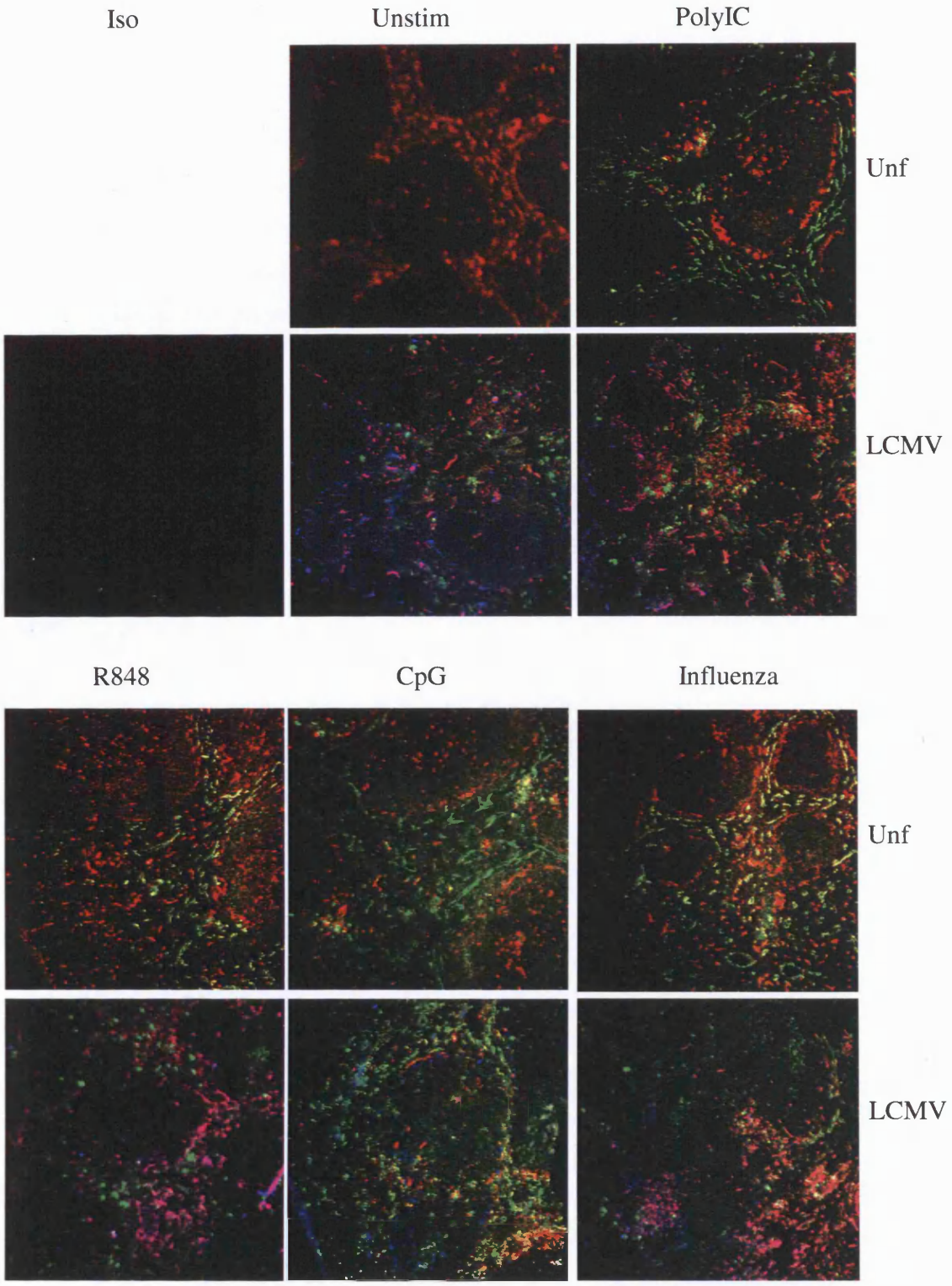

Figure 4.20 Immunofluorescent staining to identify IFN-α producing cells, LCMV-infected cells and plasmacytoid DCs in spleens from mice persistently infected with LCMV Cl13 after inoculation of innate stimuli (legend on following page)

Cryosections from the spleens of uninfected mice and mice infected LCMV Cl13 (at 3 weeks post-infection) and stimulated for 6 hours with 5 μg polyIC, 20 nmoles CpG1826 DNA, 25 nmoles R848 or 600 HA units of influenza PR8 were fixed and serially stained with the mAbs IFN-α-FITC, LCMV-NP-Cy5 and pDCA-1-biotin followed by streptavidin-Tyr-Alexa 594 to enable identification of IFN-α producing cells (green), LCMV-infected cells (blue) and plasmacytoid DCs (red). Overlapping of IFN-α and plasmacytoid DCs staining produces a yellow overlay, IFN-α and LCMV-NP113 co-staining results in a pale blue colur, LCMV-NP113 and plasmacytoid DC co-localisation results a magenta overlay, while an overlay of IFN-α, LCMV-NP113 and plasmacytoid DC stainings result in a white colour, as shown below. Appropriate isotype-matched control reagents were also tested. Images were captured at 10x magnification.

of polyIC into Cl13-infected mice were not plasmacytoid DCs, whereas at least a proportion of the cells responding to R848, CpG DNA and influenza virus inoculation by production of type 1 IFN were plasmacytoid DCs (yellow) or infected plasmacytoid DCs (white).

In summary, these results confirm the ability of mice persistently infected with LCMV to upregulate type 1 IFN production in response to inoculation of ligands for TLR 3, 7 and 9, and (less efficiently) influenza virus. Further more, they show that despite the fact that a proportion of plasmacytoid DCs in persistently infected mice are infected with LCMV, plasmacytiod DCs in these animals still participate in the type 1 IFN response elicited by TLR 7/9 ligands; and furthermore suggest that LCMV-infected plasmacytoid DCs retain the capacity to produce type 1 IFN in response to exogenous stimuli. Notably however, there was no evidence of type 1 IFN production by LCMV-infected cells other than plasmacytoid DCs at the timepoint (which would have appeared pale blue in the overlaid image).

4.11 Levels of type 1 IFN produced by ex-vivo $CD11c^+$ DCs after stimulation with TLR ligands

Results in Figure 4.14-4.17 and Figure 4.20 indicated that the extent to which type 1 IFN production was upregulated in response to TLR ligands in persistently infected mice was lower than that in uninfected mice. DCs play a key role in type 1 IFN production in response to inoculation of TLR ligands (Asselin-Paturel et al. 2001; Diebold et al. 2003) and DC numbers in persistently infected mice were reduced (Figure 4.7 and 4.20), suggesting that the impairment in mice persistently infected with LCMV Cl13 may be in part due to a reduction in the number of responding cells. To investigate whether there was also an impairment in the ability of those DC

present in persistently infected mice to respond to TLR stimuli by production of type 1 IFN, $CD11c^+$ DCs were isolated from the spleens of uninfected or mice persistently infected with LCMV Cl13 by positive selection followed by depletion of contaminating $CD11c^+$ lymphocytes as previously described. Isolated, depleted *ex vivo* DCs were stimulated with polyIC, R848 or CpG 1826 DNA and type 1 IFN activity in the supernatant was analysed after an overnight incubation (Figure 4.21). $CD11c^+$ DCs from persistently infected mice were found to produce type 1 IFN in response to all the TLR stimuli tested, although the levels produced by these cells were reduced compared to DCs from uninfected mice. Statisical analysis using the one-sample t-test showed a significant difference between levels of type 1 IFN in uninfected and LCMV-infected mice after inoculation with polyIC ($p=0.03$), R848 ($p=0.05$) and CpG DNA ($p=0.03$).

It was unclear whether the reduced type 1 IFN levels in the supernatant of TLR stimulated DCs from persistently-infected mice reflected a reduced capacity of these cells to respond to TLR ligation by type 1 IFN production and or reduced survival of these cells, which were shown to be phenotypically activated (Figure 4.8) *in vitro*. However, this result demonstrates that $CD11c^+$ DCs from LCMV Cl13-infected mice still retained the capacity to respond to the presence of external TLR stimuli.

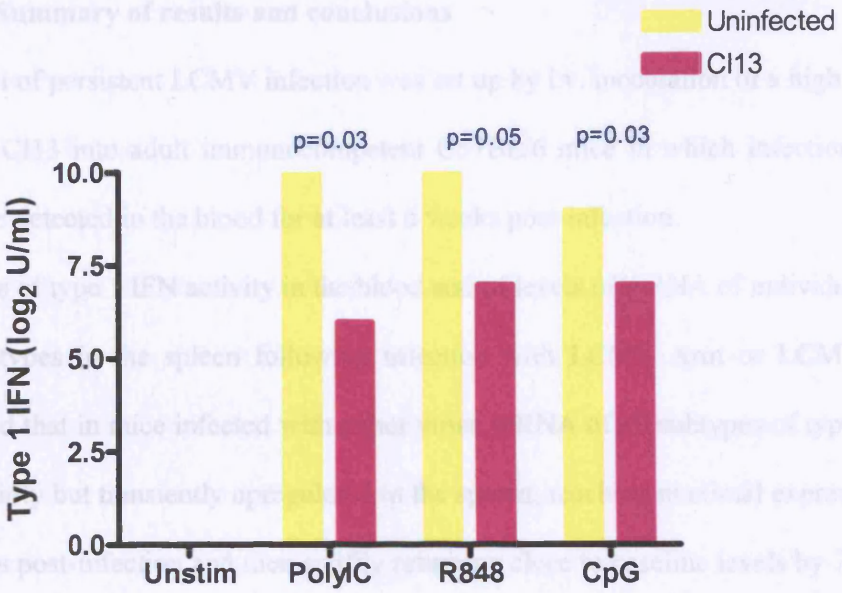

Figure 4.21 Levels of type 1 IFN produced by CD11c$^+$ DCs after *ex vivo* stimulation with TLR stimuli

CD11c$^+$ DCs isolated by positive selection and depletion of contaminating CD11c$^+$ lymphocytes from uninfected mice and mice infected with LCMV Cl13 3 weeks previously were seeded into 96-well plates at a concentration of $5x10^5$ cells/well in a 100µl volume and stimulated O/N with the following TLR ligands: polyIC at 10µg/well, R848 at 1µg/well and CpG 2216 at 0.02nmol/well. Supernatant was collected after an overnight incubation in a 37°C, 5% CO_2 incubator and the level of type 1 IFN activity was measured by antiviral bioassay. The results shown are representative of findings made in 3 independent experiments that used cells pooled from 3-10 mice per group. Statistical analysis using a one sample T-test showed a significant difference ($p<0.05$) between the levels of type 1 IFN activity induced by DCs from uninfected and Cl13-infected mice in response to stimulation with all the TLR ligands tested.

4.12 Summary of results and conclusions

A model of persistent LCMV infection was set up by i.v. inoculation of a high dose of LCMV Cl13 into adult immunocompetent C57BL/6 mice in which infectious virus could be detected in the blood for at least 6 weeks post-infection.

Analysis of type 1 IFN activity in the blood and of levels of mRNA of individual IFN-α/β subtypes in the spleen following infection with LCMV Arm or LCMV Cl13 indicated that in mice infected with either virus, mRNA of all subtypes of type 1 IFN was rapidly but transiently upregulated in the spleen; reaching maximal expression by 24 hours post-infection and then swiftly returning close to baseline levels by 72 hours post-infection. In LCMV Cl13 infected mice, very low levels of IFN-β and IFN-α4 mRNA continued to be produced in the spleen for at least 3 weeks post-infection in the context of viral persistence and ongoing high levels of viral replication. Levels of type 1 IFN activity in the blood followed a similar kinetic.

Investigation into the numbers and activation state of splenic DC subsets during persistent LCMV infection revealed that, the majority of the DCs in the spleen of persistently infected mice expressed elevated levels of MHC and co-stimulatory molecules suggesting that they were in a heightened state of activation. Notably, the number of DCs of both plasmacytoid and conventional subtypes present in the spleens of persistently infected mice were reduced compared to DC numbers in the spleens of uninfected animals, suggesting that in the context of persistent LCMV infection, the development or maturation of precursor DC was impaired and/or the lifespan of DCs was decreased (or possibly mature DCs were migrating out of the spleen to other sites). Expression of mRNAs of different subtypes of IFN-α/β was measured in plasmacytoid and conventional DC subsets at sequential timepoints after infection of mice with LCMV Arm or LCMV Cl13. In both infections, plasmacytoid DCs were

initially found to produce much higher levels of type 1 IFN on a per cell basis than conventional DCs, although the latter DC subset did contribute to type 1 IFN production. As observed in whole spleen tissue , type 1 IFN mRNA expression was very quickly downregulated in both DC subsets. In mice persistently infected with LCMV Cl13, mRNA levels were low/undetectable in both DC subsets.

The low splenic type 1 IFN mRNA levels and systemic levels of type 1 IFN in mice persistently infected with LCMV may be due in part to the low number of DCs present in the spleen and in addition, the downregulation of type 1 IFN production by DC populations remaining in the spleen may also be of importance. Infection-associated changes in the number and/or IFN-producing capacity of non-DC subsets could also be involved.

Confocal studies that visualized IFN-α, pDCA-1 from plasmacytoid DCs and LCMV-infected cells in spleen sections from mice infected with LCMV Arm or LCMV Cl13 early and late times post infection confirmed and extended these observations.

During the early stages of acute infection of mice with LCMV Arm or LCMV Cl13 splenic plasmacytoid DCs appeared activated, and some of these cells were found to be producing IFN-α, although they were not the only cells that did so. Interestingly, a high population of splenic DC appeared to be infected with LCMV Arm or LCMV Cl13.

In mice persistently infected with LCMV Cl13, plasmacytoid DC numbers in the spleen were found to be reduced. A proportion of these cells were infected with LCMV, however not all plasmacytoids DCs appeared to be infected and infection of other cell types was also observed. Low numbers of IFN-α producing cells were present in the spleen of persistently infected mice. Some (but not all) of the IFN-

producing cells were plasmacytoid DCs, and small numbers of LCMV-infected plasmacytoid DCs producing IFN-α were observed.

These studies did not aim to address the principle cellular source of type 1 IFN production in LCMV-infected mice. It remains possible that this is a non-DC cell type. Infection associated changes in the number and/or IFN-producing capacity play an important role in downregulation of systemic type 1 IFN levels both after the burst of IFN production at the acute infection and during persistent infection of mice with LCMV Cl13. However, results reported in this chapter demonstrate that type 1 IFN production is strongly upregulated in plasmacytoid DCs (and to a lesser extent conventional DCs) in the spleen very early in the acute phase of infection of mice with LCMV; and that plasmacytoid DCs are among the cell types that continue to produce small amounts of type 1 IFN in mice persistently infect with LCMV Cl13. The low splenic type 1 IFN mRNA levels in the spleens of mice persistently infected with LCMV may be due in part to the number of DCs present in the spleen of these animals being decreased relative to the numbers present in uninfected mice, but low type 1 IFN production by the DC remaining in the spleen could also be a contributing factor. Evidence of infection of DCs by LCMV was obtained and this may be involved in impairment of type 1 IFN production.

Experiments carried out to address the ability of mice persistently infected with LCMV to produce type 1 IFN in response to inoculation of synthetic ligands for a selection of virally-important TLRs revealed that these stimuli did elicit upregulation of type 1 IFN mRNA and protein production in mice persistently infected with LCMV, although the upregulation observed was lower than that seen in uninfected mice. Lower levels of IL-12 and IFN-γ were also produced in persistently infected mice, although production of other cytokines was evaluated. *Ex-vivo* stimulation of

purified $CD11c^+$ splenic DCs from persistently infected mice with TLR ligands provided further evidence that these cells could respond (albeit less well than their uninfected counterparts) to TLR stimuli by IFN-α/β production. In contrast, when mice were injected i.p. with live influenza PR8, they produced much less type 1 IFN than uninfected mice. Interpretation of this observation was complicated by the fact that although influenza viral RNA was detected in the spleen of both groups of mice, levels were higher in the uninfected mice suggesting they may have experienced a heavier burden of infection. Nonetheless, it is tempting to speculate that the ability of LCMV-infected mice to respond to infection with RNA virus by production of type 1 IFN is impaired A possible explanation for those results is that persistent LCMV infection may impair the RNA helicase-mediated intracellular innate viral recognition pathway while leaving the TLR-mediated type 1 IFN induction pathway intact.

The following chapter (5) described *in vitro* experimental work that was carried out to gain more insight into type 1 IFN production by LCMV-infected cells or mechanisms that may underlie impairments in their responsiveness.

Results from Chapter 3 and Chapter 4 are then discussed together in Chapter 5.

CHAPTER 5

ANALYSIS OF THE MECHANISMS INVOLVED IN THE DOWNREGULATION OF TYPE 1 IFN PRODUCTION DURING PERSISTENT LCMV Cl13 INFECTION

5.1 Introduction

Observations from *in vivo* experiments indicated that there was only a very low level of ongoing type 1 IFN production in mice persistently infected with LCMV, despite the presence of high levels of infection in the host. Persistently-infected animals retained the capacity to upregulate type 1 IFN in response to exogenous TLR stimuli, as type 1 IFNs and other cytokines were produced following i.p. inoculation of TLR3, 7 and 9 ligands. However, their capcity to upregulate type 1 IFN production via intracellular pathways triggered by RNA virus replication was less clear. There may be several reasons why type 1 IFNs are not continuously produced at higher levels in mice persistently infected with LCMV Cl13. Although there are large numbers of infected cells in these animals, the level of infectious virus they secrte may be relatively low and their turnover may be slow resulting in the levels of TLR ligands (e.g. ssRNA, ds replicative RNA and possibly viral glycoproteins) available for TLR stimulation being relatively low. It is also possible that host cells may be de-sensitised to TLR stimuli that are constitutively present, but retain the capacity to respond to a rapid elevation in the level of TLR ligands in their environment. The reduction in splenic plasmacytoid DC and conventional DC numbers in mice persistently infected with LCMV may also play a role. Coupled with this, there may be defect(s) in the pathway(s) for recognition of intracellular RNA virus replication and/or upregulation of type 1 IFN production in response to this in cells infected with LCMV.

These defects could be specific to the intracellular RNA recognition response pathway so that infected cells are still able to recognize and respond to external innate stimuli (accounting for the observation of some LCMV-infected IFN-α producing cells- principally plasmacytoid DCs – in persistently infected mice). The defects in this pathway could be induced by the virus as a strategy for avoiding control by the host immune response or may instead constitute a host response to limit immunopathology mediated damage mediated by high levels of type 1 IFN and other inflammatory cytokines in the system.

Efforts to use *in vivo* studies to address mechanism(s) that may be involved in impairment of type 1 IFN production during LCMV infection are hampered by the complexity of the *in vivo* system. There are multiple heterogenous cell-types present which differ in their capacity to produce type 1 IFNs and pathways for triggering of IFN production. The cell types producing type 1 IFNs in LCMV-infected mice and how these parameters change over time are all incompletely understood. Further, the extent to which different cell types are infected by LCMV, and the changes taking place in numbers and proportions of cell subsets over the course of infection are difficult to discern. In addition, the levels and relative proportions of type 1 IFN-inducing stimuli present are hard to quantify. In this chapter, I thus used *in vitro* systems to study the effects of LCMV infection on the ability of homogenous cell populations to produce type 1 IFNs in response to different IFN-inducing stimuli.

Many strains of LCMV have been reported to infect primary cells *in vitro*. Infection of most cell types is largely non-cytopathic, and the cells typically grow normally following infection, exhibiting a generation time and cloning efficiency identical to that of uninfected cells (Staneck et al. 1972). Importantly, once cells are infected with LCMV *in vitro*, the infection persists in the culture over time. All cells are infected with virus, as

demonstrated by immunofluorescence staining for LCMV proteins in cultures of cells infected for long periods, although the level of infectious virus released into the supernatant is often relatively low, which has been associated with downregulation of expression of the viral glycoproteins in persistently-infected cells (Fuller-Pace and Southern 1988).

Cell lines persistently infected with LCMV provide an excellent *in vitro* system for analysing the effects of LCMV infection on the ability of cells to produce type 1 IFNs, and addressing the mechanisms involved in dysregulation of IFN production. Using this approach, the response of a single cell type with a defined TLR ligand response profile to different IFN-inducing stimuli can be analysed, and the effects of persistent LCMV infection on the type 1 IFN response addressed using a population of cells, all of which are infected with virus. I thus planned to persistently infect different types of cell lines (fibroblast and dendritic cells) with LCMV, and compare the ability of uninfected and infected cells to produce type 1 IFNs in response to stimulation via TLRs and the intracytoplasmic helicase-dependent pathways for detection of RNA virus replication. If LCMV infection was found to impair the ability of cells to produce type 1 IFNs in response to any of these stimuli, a further objective was to investigate the role of different LCMV proteins in mediating this effect by generating cell lines stably transfected with individual LCMV proteins (GP, NP or Z) and examining whether they showed a similar defect in type 1 IFN production.

5.2 Analysis of the type 1 IFN response induced following acute infection of BM-DCs with LCMV

BM-DCs are primary cells matured from bone marrow precursor cells by GM-CSF that have a number of properties in common with conventional DCs subsets found in the

spleen (Boonstra et al. 2003) and have been previously reported to produce type 1 IFN when infected with HSV virus (Hochrein et al. 2004). Prior to carrying out experiments with long-term cell lines, an initial *in vitro* experiment was thus carried out using BM-DCs, to analyse the levels of type 1 IFN mRNA and activity induced when a population of cells are acutely infected with LCMV.

5.2.1 Infectious viral titres

BM-DCs were prepared as described in Section 2.8.1. The cells were cultured in the presence of polymyxin B in order to limit the possibility that type 1 IFN was induced by endotoxin contamination. Following a 6-day differentiation period, BM-DCs were infected with LCMV Arm or LCMV Cl13 at m.o.i. of 5. This high m.o.i. was selected to try to achieve initial infection of the vast majority of cells, so that the response of cells to infection could be studied. The morphology of the cells was found to alter following infection: they displayed characteristics of activated DC, such as increasing in size, acquiring dendrites and migrating into suspension. While LCMV usually causes a non-cytopathic infection, cells infected with LCMV Cl13 seemed to be dying shortly after exposure to the virus. It was not clear whether this was a direct result of infection with LCMV as even under non-infectious circumstances, BM-DC cells have a very short lifespan that is reduced following activation. Notably, BM-DCs infected with LCMV Arm seemed to survive better than those infected with LCMV Cl13. Cl13 is reported to infect $CD11c^+$ cells more efficiently than LCMV Arm (Sevilla et al. 2000), hence reduced survival of LCMV Cl13-infected cultures could be a reflection of larger numbers of cells becoming infected and apoptosing as a result of infection and/or associated activation.

Kinetic analysis of the levels of infectious virus secreted in the supernatant after an infection with a high dose of LCMV Arm or Cl13 is shown in Figure 5.1. By 24 hours

post-infection, the infectious titre of LCMV in the supernatant of the LCMV Cl13-infected cells was a log higher than that in the LCMV Arm-infected cells.

5.2.2 Analysis of levels of type 1 IFN and Mx mRNA by Taqman qRT-PCR

Having determined that the cells were infected with LCMV, the type 1 IFN response to the presence of the infection was measured by real-time Taqman qRT-PCR over time post-infection. As depicted in Figure 5.2, quantitative real time RT-PCR analysis of total RNA purified from the infected/polyIC stimulated BM-DCs over time showed that although IFN-α mRNA levels remained fairly stable over time in uninfected cells, higher levels of IFN-β were detected at the first timepoint analysed (6 hours) than at late timepoints, suggesting that manipulation of the BM-DCs (such as harvesting and plating out) may have itself induced an upregulation of type 1 IFN production. This has been observed before: simply pipetting GM-CSF matured BM-DCs into fresh plates may activate the cells (Gallucci et al. 1999). In polyIC stimulated BM-DCs, there was strong upregulation in the levels of IFN-β and IFN-α subtypes at 6 hours post-infection, substantially greater than that observed in untreated cells; this was followed by a slow decline in the mRNA of all subtypes of IFN, reacing baseline levels between 48 and 72 hours post-stimulation. By contrast, there was a much less dramatic increase in type 1 IFN production in cells infected with LCMV. In Arm-infected cells, the levels peaked at ~12 hours post-infection, the relatively delayed kinetics (relative to polyIC-stimulated cells) possibly reflecting infection of cells and accumulation of viral components over time. However, maximal type 1 IFN mRNA levels in LCMV Cl13-infected cells were recorded at 6 hours post-infection – this could be a consequence of high levels of apoptosis that

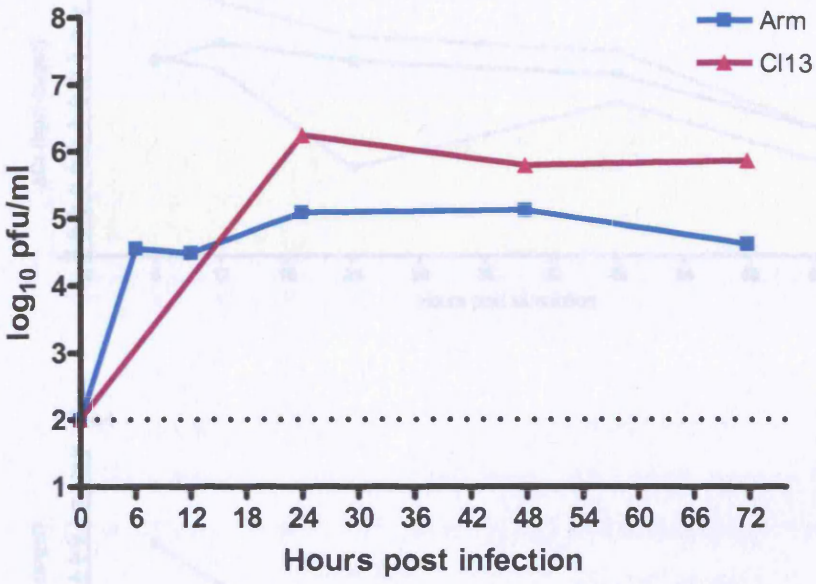

Figure 5.1 Kinetic analysis of the levels of infectious virus in the supernatant of BM-DCs infected with LCMV Arm or Cl13

BM-DCs were infected at an m.o.i. of 5 with either LCMV Arm or Cl13 and plated out at $2x10^6$cells into 60 mm dishes. Supernatant was collected from single wells at the indicated timepoints. Infectious viral titres were determined by viral plaque assay and are expressed as plaque forming units per ml of supernatant (p.f.u/ml). The dotted line indicates the limit of sensitivity for this assay, which was $1x10^2$ p.f.u/ml. The results shown are representative of three independent experiments.

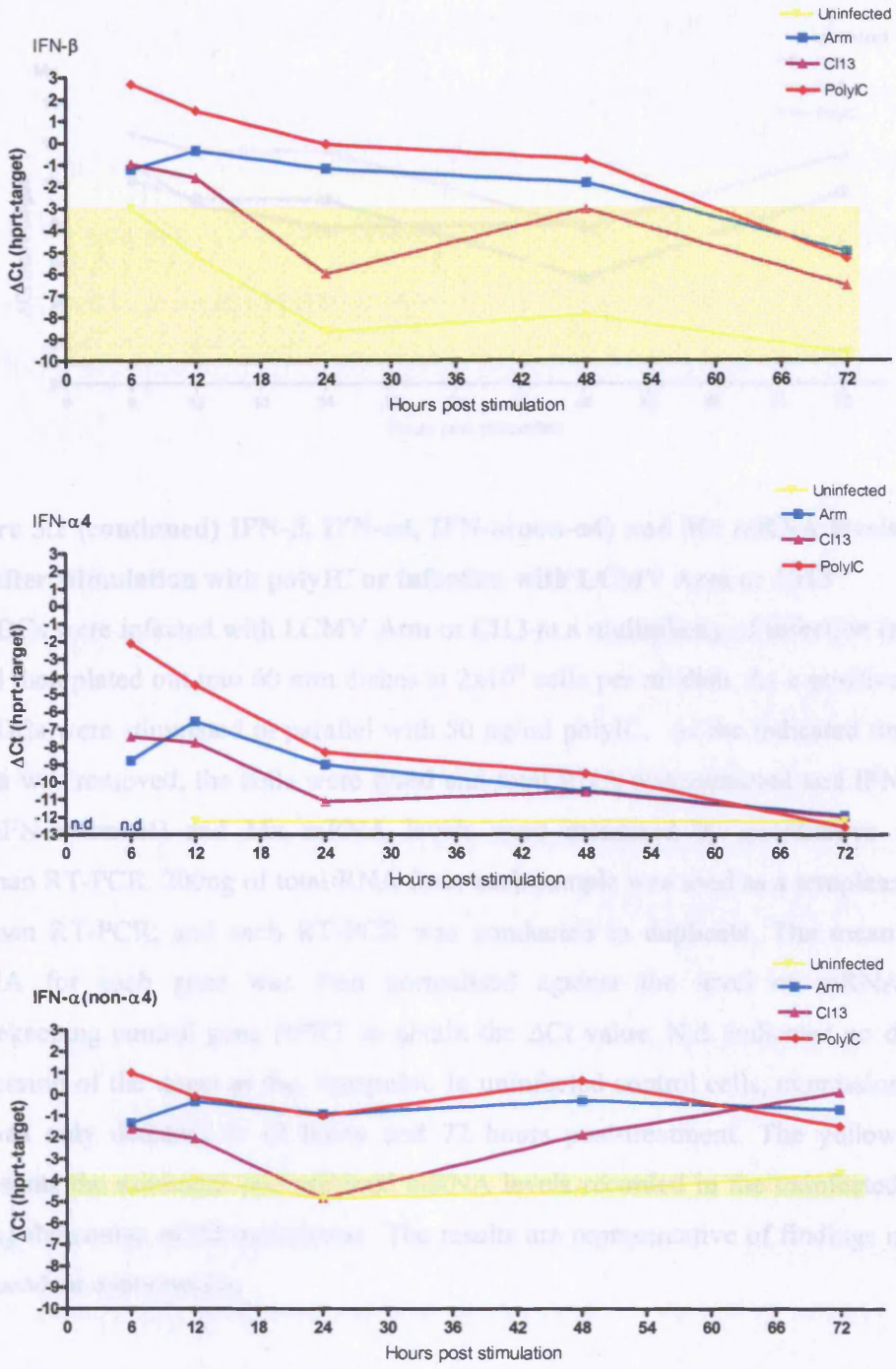

Figure 5.2 IFN-β, IFN-α4, IFN-α(non-α4) and Mx mRNA levels in BM-DC after stimulation with polyIC or infection with LCMV Arm or Cl13 (figure continued on following page)

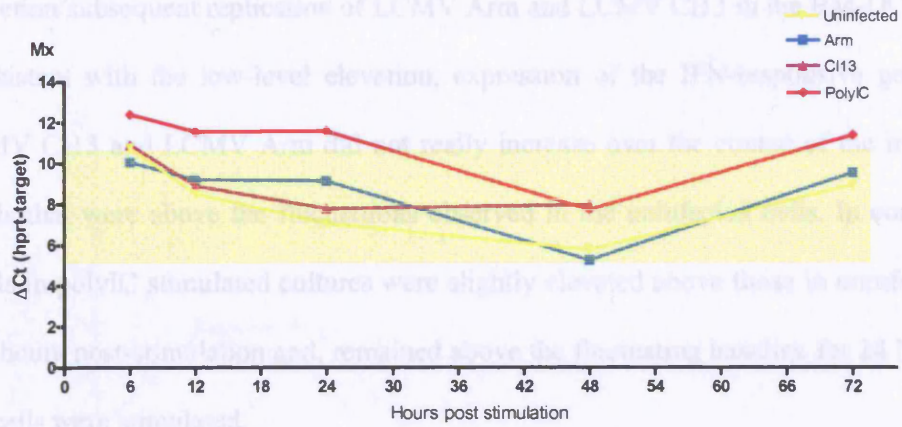

Figure 5.2 (continued) IFN-β, IFN-α4, IFN-α(non-α4) and Mx mRNA levels in BM-DC after stimulation with polyIC or infection with LCMV Arm or Cl13

BM-DCs were infected with LCMV Arm or Cl13 at a multiplicity of infection (m.o.i.) of 5 and then plated out into 60 mm dishes at $2x10^6$ cells per ml/dish. As a positive control, BM-DCs were stimulated in parallel with 50 μg/ml polyIC. At the indicated timepoints, media was removed, the cells were lysed and total RNA was extracted and IFN-β, IFN-α4, IFN-α(non-α4) and Mx mRNA levels were measured by quantitative real-time Taqman RT-PCR. 200ng of total RNA from each sample was used as a template for each Taqman RT-PCR; and each RT-PCR was conducted in duplicate. The mean level of mRNA for each gene was then normalised against the level of mRNA of the housekeeping control gene HPRT to obtain the ΔCt value. N.d. indicates no detectable expression of the target at that timepoint. In uninfected control cells, expression of IFN-α4 was only detected at 12 hours and 72 hours post-treatment. The yellow shading represents the minimum and maximal mRNA levels recorded in the uninfected controls during the course of the experiment. The results are representative of findings made in 2 independent experiments.

occurred in the cultures or it may have reflected differences in the level of initial infection/subsequent replication of LCMV Arm and LCMV Cl13 in the BM-DCs.

Consistent with the low-level elevation, expression of the IFN-responsive gene Mx in LCMV Cl13 and LCMV Arm did not really increase over the course of the infection to levels that were above the fluctuations observed in the uninfected cells. In contrast, Mx levels in polyIC stimulated cultures were slightly elevated above those in uninfected cells at 6 hours post-stimulation and, remained above the fluctuating baseline for 24 hours after the cells were stimulated.

5.2.3 Analysis of type 1 IFN activity in the supernatant

The levels of type 1 IFN activity observed in the supernatant over time, shown in Figure 5.3, fits very well with the results of the mRNA analysis. The supernatant was collected at the same time as the cells were harvested for RNA extraction and so the levels represent the level of type 1 IFN that had accumulated in the supernatant by each timepoint. Type 1 IFN activity was detected in the supernatant of uninfected cells at the first timepoints analysed, but levels were not sustained over time. Stimulation with polyIC induced a marked elevation in supernatant type 1 IFN activity which peaked at 12 hours post-stimulation and remained elevated throughout the course of the experiment. There was a modest elevation in type 1 IFN activity in the supernatant of LCMV Arm and Cl13 infected cultures which peaked at 24 hours post-infection before falling to baseline in the next 24 hours. The level of activity induced by LCMV Arm and LCMV Cl13 infection was similar, although IFN levels in the supernatant of LCMV Arm-infected cells were sustained for slightly longer than those in the supernatant of cells infected with LCMV Cl13. The results here were largely in agreement with data obtained from real-time Taqman quantitative RT-PCR, with the delay in the peak activity recorded at 12-24 hours

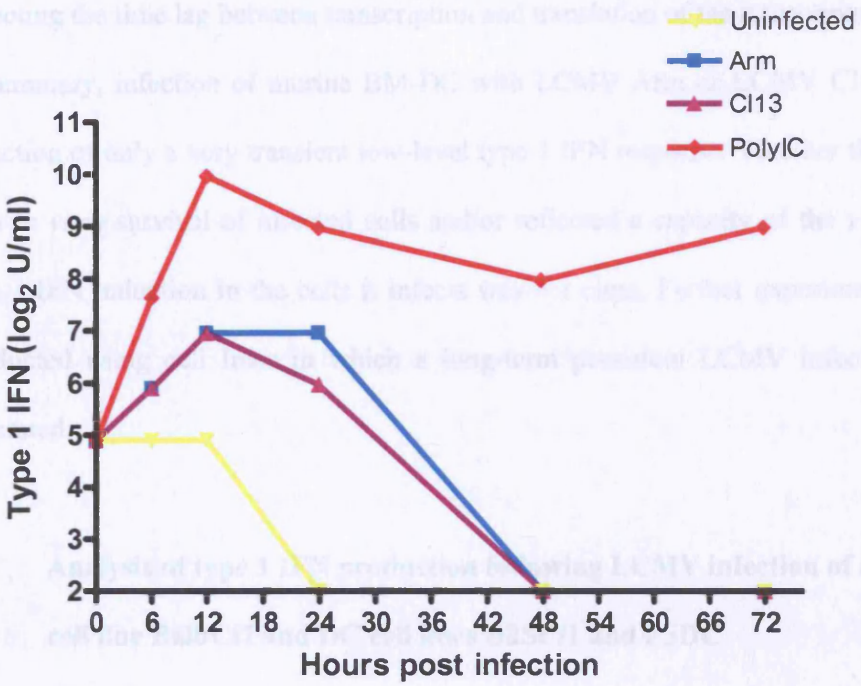

Figure 5.3 Type 1 IFN activity in the supernatant of BM-DC cultures after infection with LCMV or stimulation with polyIC

BM-DC at $2x10^6$ cells in 60 mm dishes were infected at a m.o.i. of 10 with LCMV Arm or LCMV Cl13 or stimulated with 50 μg/ml polyIC. Supernatant was collected from different wells at the timepoints indicated and type 1 IFN activity was measured by bioassay. IFN activity was quantitated by comparison with a recombinant IFN-α standard curve; the results shown are expressed in log_2 units/ml (U/ml). The results shown are representative of 2 independent experiments.

post-infection as opposed to 6 hours post-infection for the mRNA transcripts, probably reflecting the time lag between transcription and translation of the transcripts.

In summary, infection of murine BM-DC with LCMV Arm or LCMV Cl13 resulted in induction of only a very transient low-level type 1 IFN response. Whether this was due to poor *in vitro* survival of infected cells and/or reflected a capacity of the virus to impair type 1 IFN induction in the cells it infects was not clear. Further experiments were thus conducted using cell lines in which a long-term persistent LCMV infection could be generated.

5.3 Analysis of type 1 IFN production following LCMV infection of the fibroblast cell line BalbCl7 and DC cell lines D2SC/1 and FSDC

5.3.1 Infectious viral titres

BM-DC do not survive for long in culture, maturing and undergoing apoptosis once they become infected, which renders them unsuitable for long term analysis of the effects of persistent LCMV infection on the type 1 IFN response. I thus carried out further experiments using an immortalized fibroblast cell line, BalbCl7, which was adherent and known to be susceptible to infection by LCMV; and two DC lines, D2SC/1 and FSDC which had previously been shown to produce measurable levels of type 1 IFN upon infection infection with Sendai, another virus with an RNA genome (Eloranta et al. 1997). The DC lines were semi-adherent, and it was not clear how readily they would be infected by LCMV. Initial experiments were thus carried out in which BalbCl7, D2SC/1 and FSDC cells were infected with low or high doses of LCMV Cl13 and the proportion of infected cells and virus release into the supernatant analysed by immunofluorescent staining and viral plaque assays respectively.

Immunofluorescent staining of the three cell lines with LCMV-specific antibodies showed that 24 hours following infection with LCMV Cl13 at a high m.o.i., viral antigens could be detected in the vast majority of cells, indicating that they were heavily infected with virus (Figure 5.4). When infected at a m.o.i. of 0.1, viral antigens could be detected at 24 hours in ~20% of BalbCl7 cells and ~45-55% of cells of DC lineage, indicative of a higher initial level of infection and/or greater viral production or faster spread in the latter cells. However, by 48 hours post-infection, in BalbCl7, D2SC/1 and FSDC cultures, the vast majority of cells were all heavily infected with LCMV. Viral plaque assays on cell supernatants confirmed that all three cell lines had been infected with LCMV and produced infectious virus particles following infection (Figure 5.4). The magnitude of kinetics of infectious virus production in BalbCl7 cells and the two DC cell lines differed slightly with peak viral titres in BalbCl7 cell supernatant being higher than those in the supernatant of the DC cell lines and being reached somewhat later (2-3 rather than 1-2 days post-infection). In all three cell lines, infection at low m.o.i. produced higher peak viral titres than infection at high m.o.i., with viral titres also peaking more rapidly after infection at the higher m.o.i. Infection of all three cell lines with LCMV Cl13 at either low or high m.o.i. resulted in long-term virus persistence with continued production of infectious virus into the cell supernatant (Figure 5.5). Immunofluorescent staining of persistently infected cells (Figure 5.6) confirmed expression of the LCMV-NP protein in the vast majority of cells, indicating that the entire population was infected.

A) BalbCl7

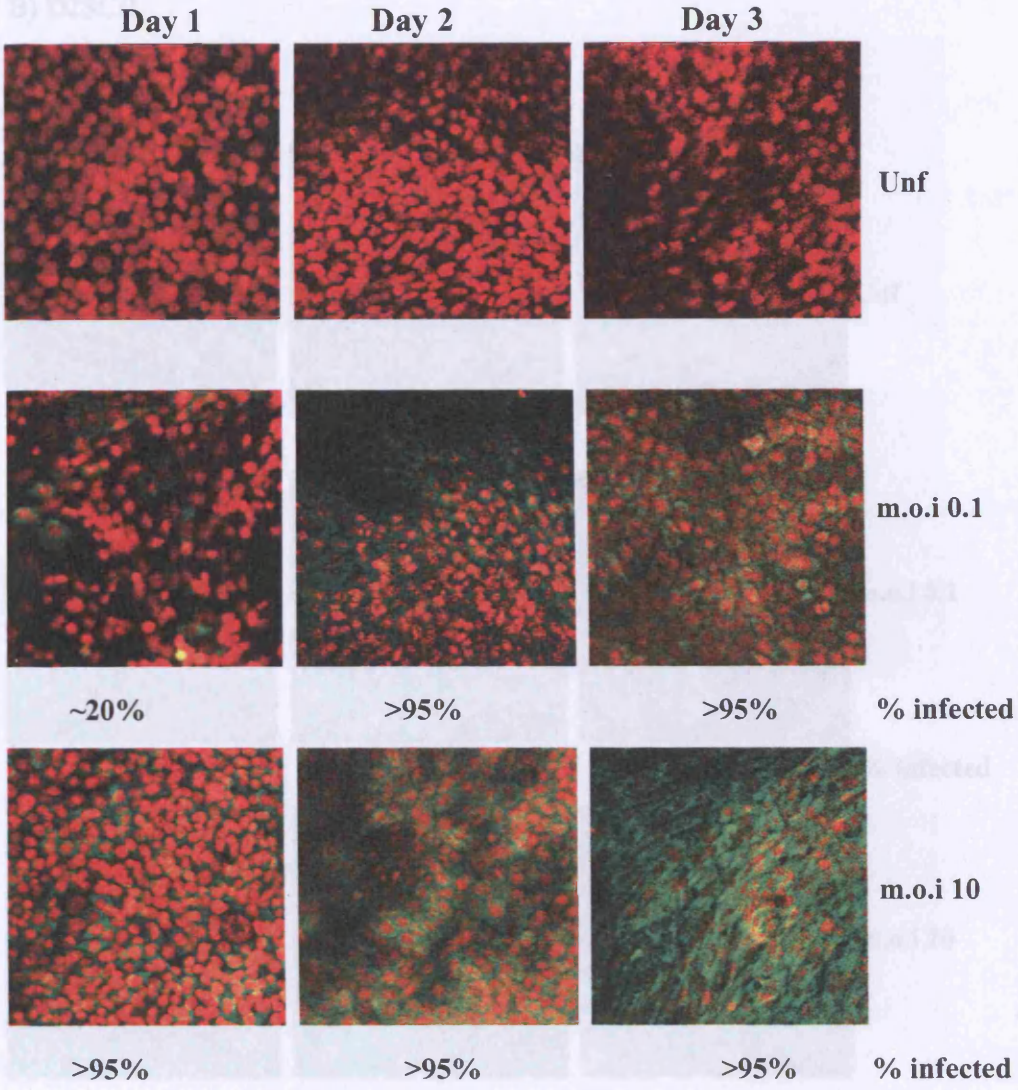

Figure 5.4 Expression of LCMV proteins in BalbCl7, D2SC/1 and FSDC cells over time following infection with LCMV Cl13 at low and high m.o.i. (figure continued on following pages)

B) D2SC/1

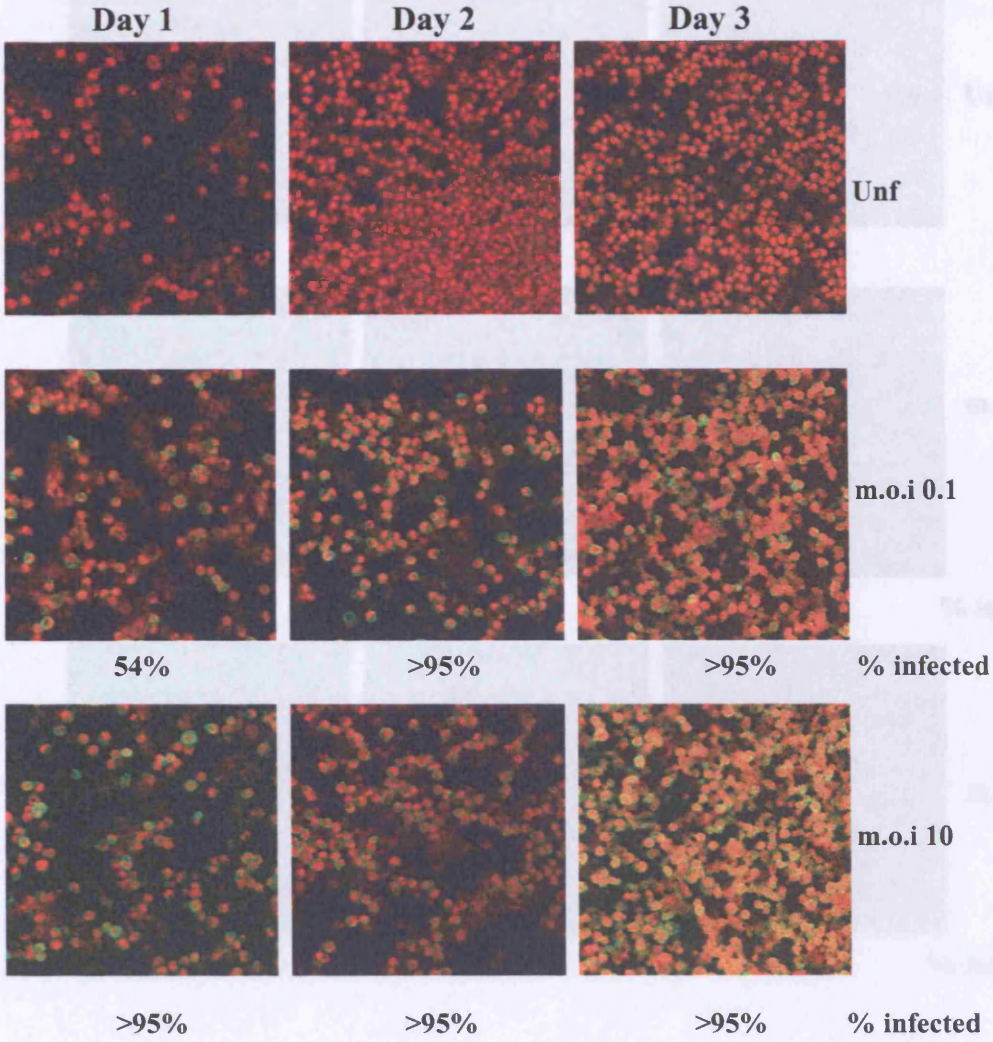

Figure 5.4 (continued) Expression of LCMV proteins in BalbCl7, D2SC/1 and FSDC cells over time following infection with LCMV Cl13 at low and high m.o.i. (figure continued on following pages)

C) FSDC

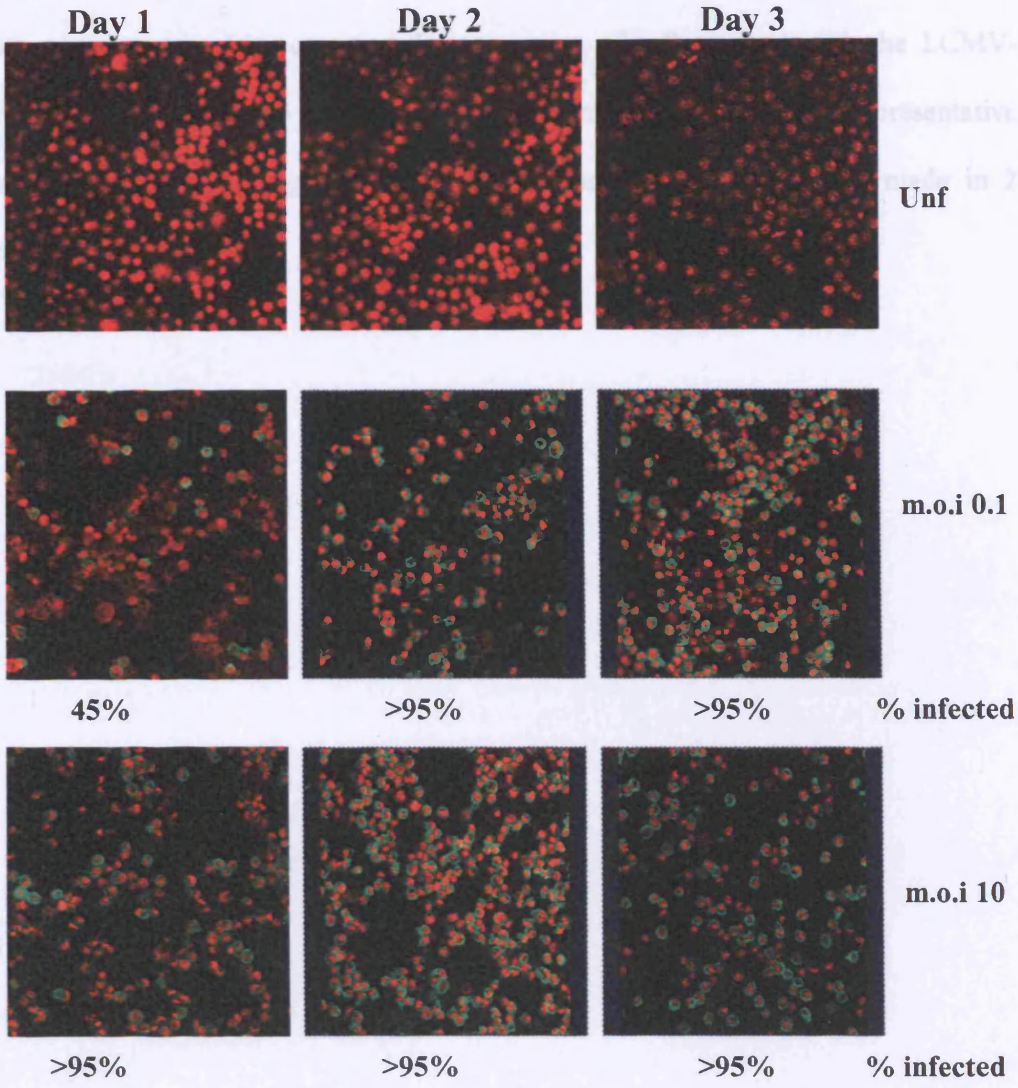

Figure 5.4 (continued) Expression of LCMV proteins in BalbC17, D2SC/1 and FSDC cells over time following infection with LCMV Cl13 at low and high m.o.i.

A fibroblast cell line, BalbC17 and two DC cell lines, D2SC/1 and FSDC, were infected at low (0.1) and high (10) m.o.i.s with LCMV Cl13. Infected cells were either plated out on Lab-Tek chamber slides (BalbC17) or spotted onto wells on a Teflon slide (D2SC/1 and FSDC). At the indicated times post-infection, cells were fixed, and the proportion of cells infected with LCMV was determined by staining with ascitic fluid containing LCMV-

specific antibodies (VR164) at a 1/50 dilution followed by anti-mouse Ig-FITC (green). Cell nuclei were stained with Bo-Pro (red). The percentage of infected cells in each sample was determined by counting the proportion of cells stained with the LCMV-specific antibody in at least 3 fields. The results shown here are pictures of representative fields visualised at 10x magnification, and are representative of findings made in 2 independent experiments.

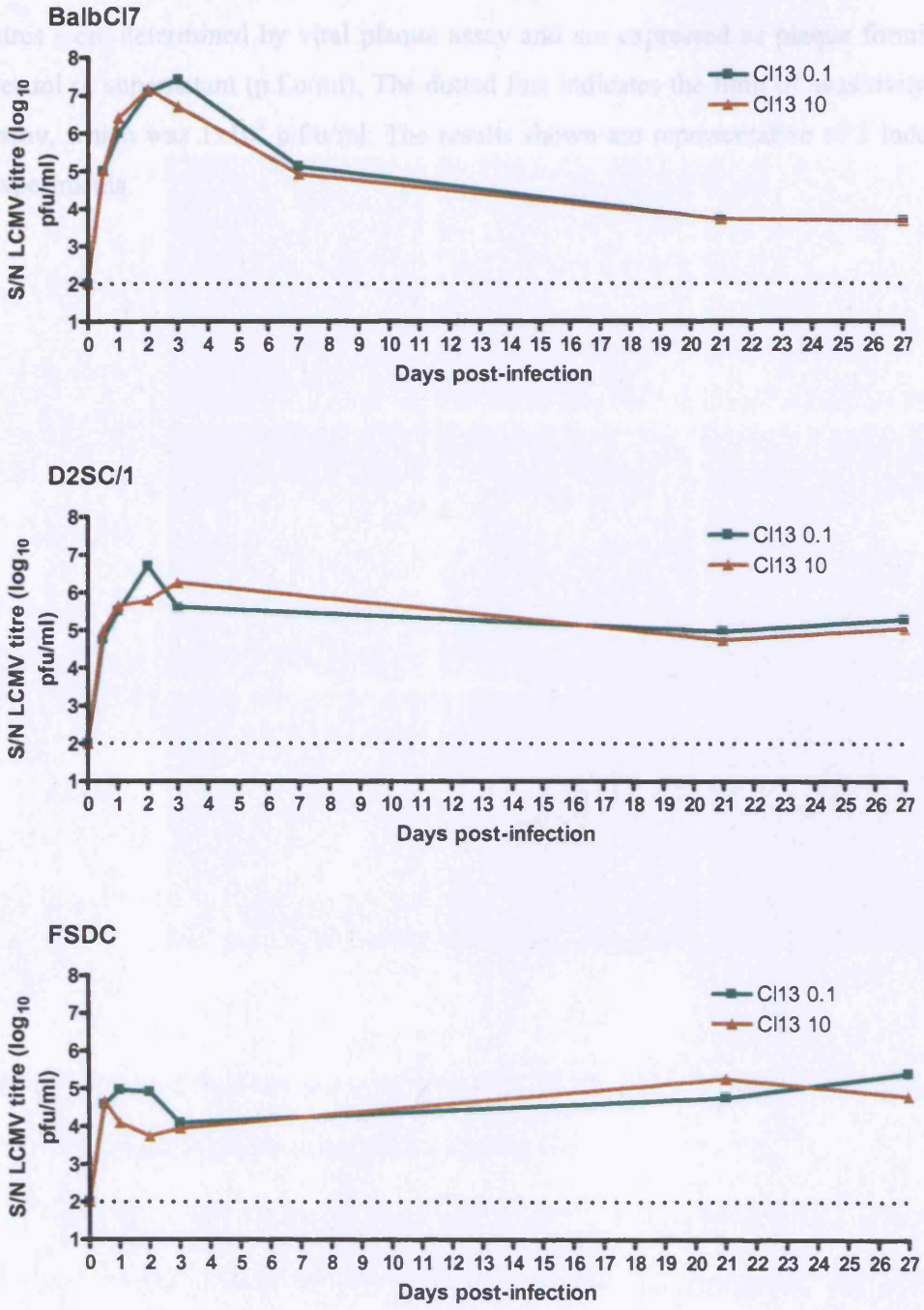

Figure 5.5 Kinetic analysis of the levels of infectious virus in the supernatant of BalbCl7, D2SC/1 and FSDC cells infected with low or high doses of LCMV Cl13

BalbCl7, D2SC/1 and FSDC cells were infected at an m.o.i. of 0.1 or 10 with LCMV Cl13 and were seeded out into 6-well dishes at $1x10^6$ cells per well in a 1 ml volume. Supernatant was collected from single wells at the indicated timepoints. Infectious viral

titres were determined by viral plaque assay and are expressed as plaque forming units per ml of supernatant (p.f.u/ml). The dotted line indicates the limit of sensitivity for this assay, which was $1x10^2$ p.f.u/ml. The results shown are representative of 2 independent experiments.

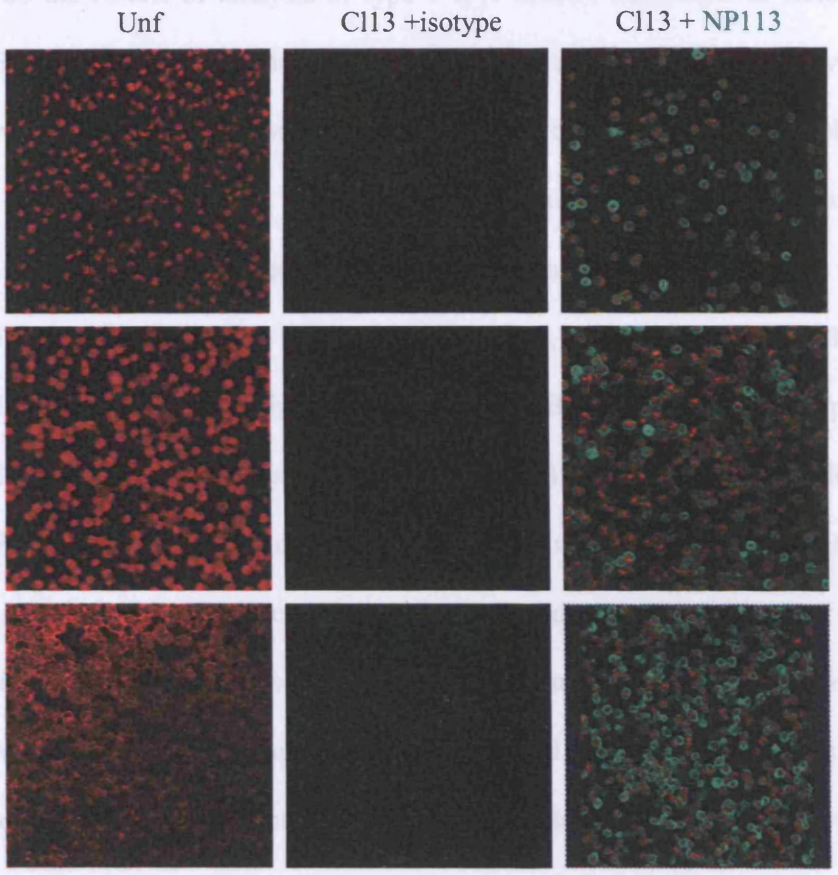

Figure 5.6 Immunofluorescent staining for LCMV NP in BalbCl7, D2SC/1 and FSDC cells persistently infected with LCMV Cl13

Cells which were persistently infected with LCMV Cl13 were spotted onto Teflon slides, fixed and stained with monoclonal antibody NP113 (that recognizes the LCMV NP protein) followed by anti-mouse IgG-FITC (green). Cell nuclei were stained with Bo-Pro (red). The percentage of infected cells in each sample was determined by counting the proportion of cells stained with the LCMV-specific antibody in at least 3 fields. The results shown here are pictures of representative fields visualised at 10x magnification, and are representative of findings made in 2-4 independent experiments.

5.3.2 mRNA and activity of Type 1 IFN in persistent LCMV infection

Figure 5.7 shows the results of analysis of type 1 IFN mRNA transcripts in BalbC17, D2SC/1 and FSDC cells at different times following infection with LCMV C113. Type 1 IFN mRNA levels in uninfected cells were found to fluctuate over time in all three cell lines possibly reflecting a response to manipulation/medium change during passage and/or changes in the degree of confluence of cells. LCMV infection was associated with very little type 1 IFN mRNA above the background range, with a transient low-level increase in IFN-β and IFN-α4 transcripts being observed in BalbC17 cells 1-2 days post-infection, and little response being detected in the DC cell lines. In all three cell lines, type 1 IFN mRNA levels in persistently-infected cells did not differ appreciably from those observed in uninfected cells. These observations were supported by analysis of type 1 IFN activity in the supernatant shown in Figure 5.8. Fluctuating (but fairly low) levels of type 1 IFN were detected in the supernatant of uninfected cells over time, and it was very difficult to distinguish any infection-associated upregulation in supernatant type 1 IFN levels above this backround in any of the cell lines tested. In both fibroblast and DC cell lines, it thus appeared that infection with LCMV induced low to negligible levels of type 1 IFN production.

5.4 Levels of Type 1 IFN induced by different viral infections or polyIC

As shown in Figures 5.7 and 5.8, LCMV infection of BalbC17, D2SC/1 or FSDC cells was associated with little type 1 IFN induction. In order to determine whether this was due to abnormalities in these cell lines which were immortalized in order to be amenable to culture and may have potentially lost the ability to recognize/respond to infectious stimuli by production of type 1 IFN, or reflected a specific failure to upregulate type 1 IFN production upon infection with LCMV, possibly dues to virus-induced impairment of

A) BalbCl7

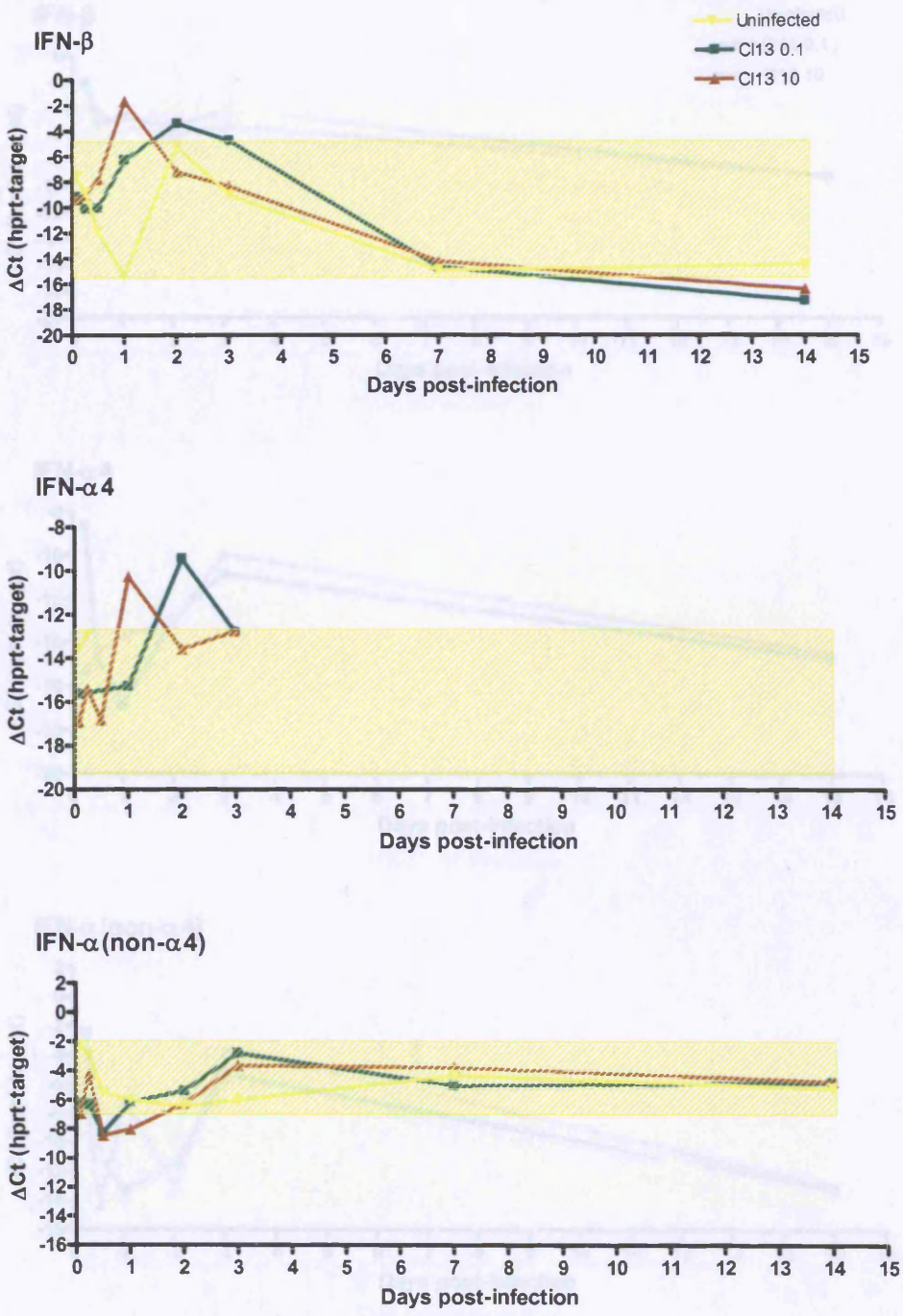

Figure 5.7 Levels of IFN-β, IFN-α4 and IFN-α(non-α4) mRNA in BalbCl7, D2SC/1 and FSDC cells after infection at low or high m.o.i. with LCMV Cl13 (figure continued on following pages)

B) D2SC/1

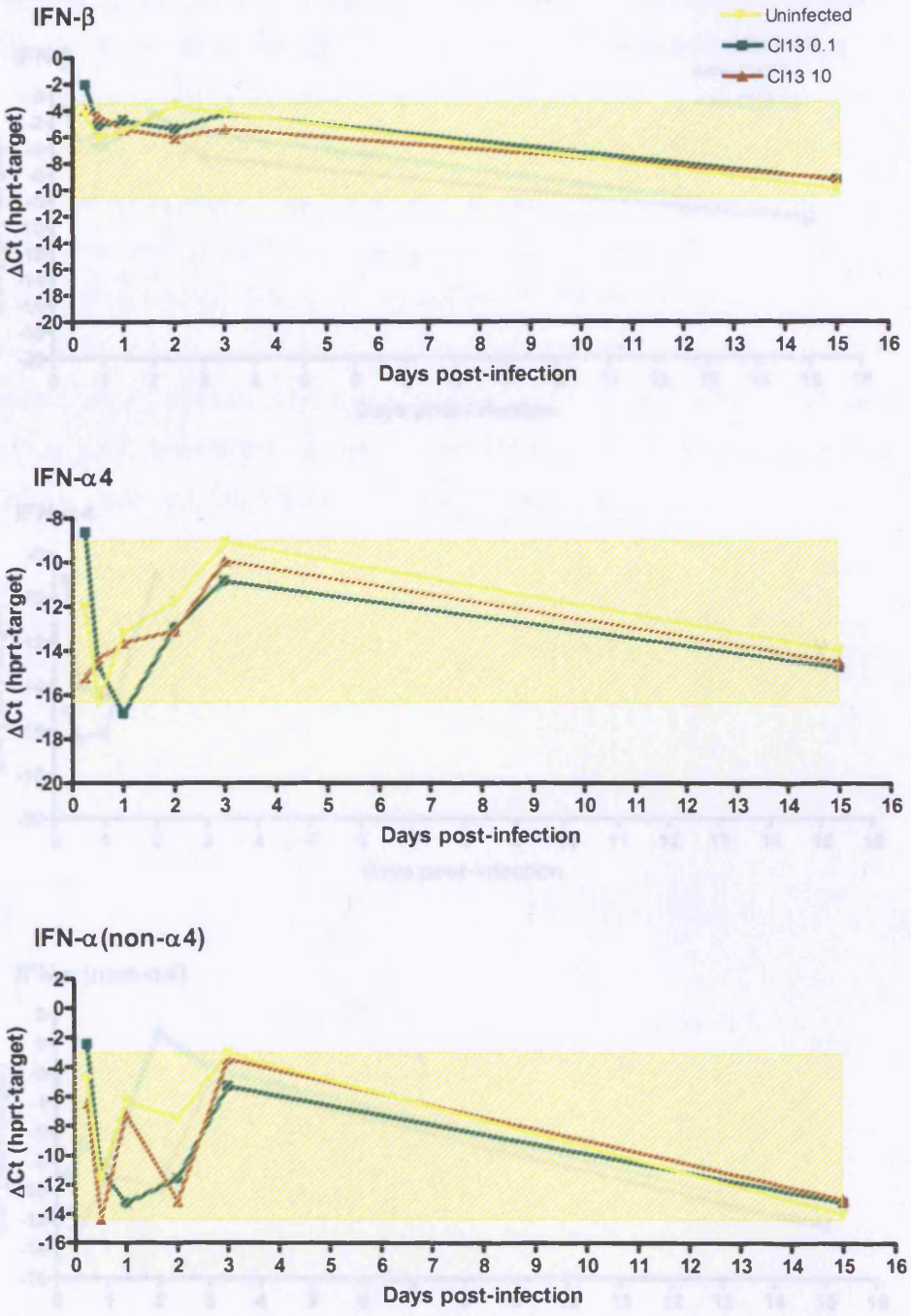

Figure 5.7 (continued) Levels of IFN-β, IFN-α4 and IFN-α(non-α4) mRNA in BalbCl7, D2SC/1 and FSDC cells after infection at low or high m.o.i. with LCMV Cl13 (figure continued on following pages)

C) FSDC

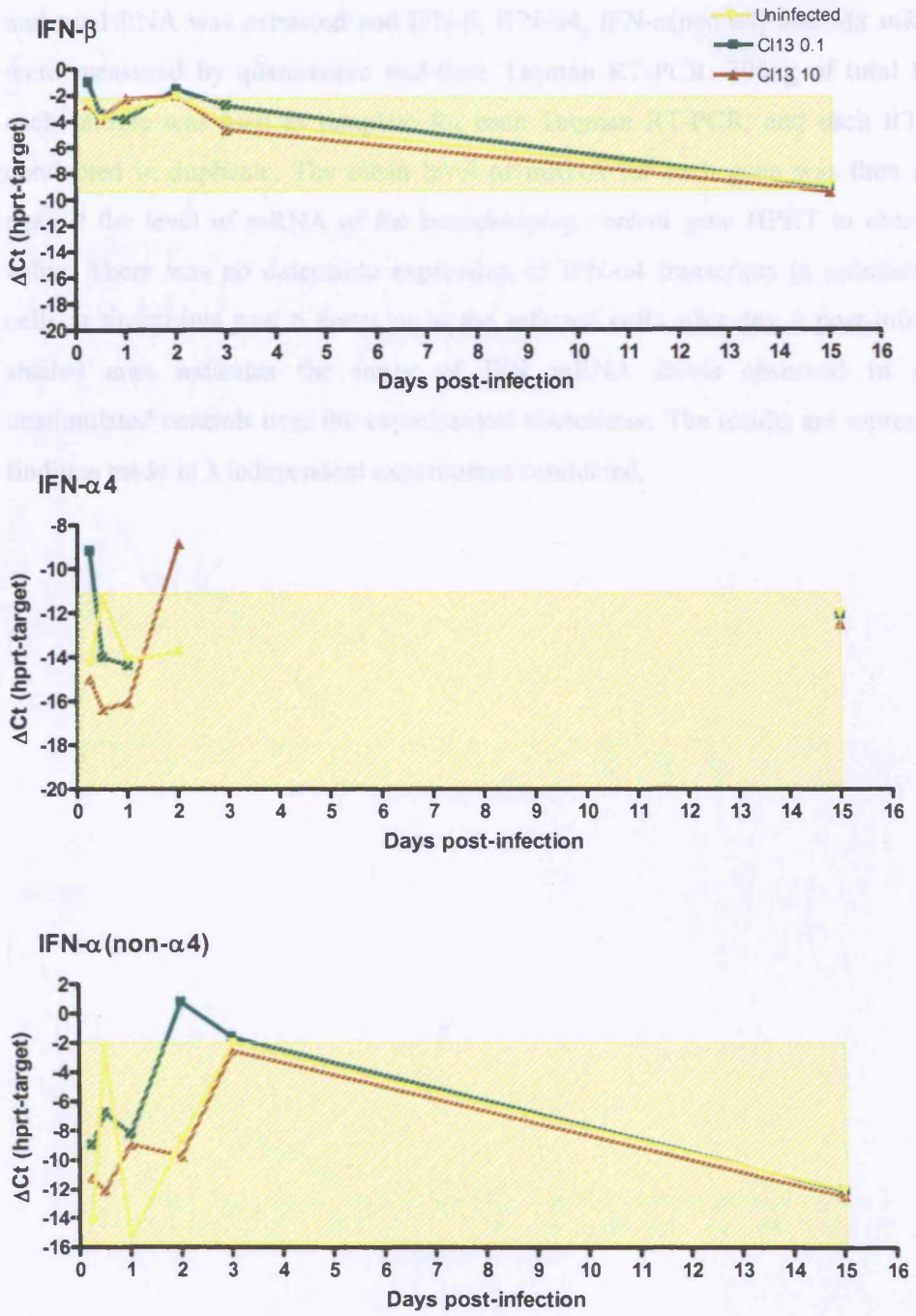

Figure 5.7 (continued) Levels of IFN-β, IFN-α4 and IFN-α(non-α4) mRNA in BalbCl7, D2SC/1 and FSDC cells after infection at low or high m.o.i. with LCMV Cl13

BalbCl7, D2SC/1 or FSDC cells were infected with LCMV Cl13 at a multiplicity of infection (m.o.i.) of 0.1 or 5 and then seeded into 6-well dishes at $1x10^6$ cells/ml per well

in a 1 ml volume. At the indicated timepoints, medium was removed, the cells were lysed and total RNA was extracted and IFN-β, IFN-α4, IFN-α(non-α4) and Mx mRNA levels were measured by quantitative real-time Taqman RT-PCR. 200ng of total RNA from each sample was used as template for each Taqman RT-PCR; and each RT-PCR was conducted in duplicate. The mean level of mRNA for each gene was then normalised against the level of mRNA of the housekeeping control gene HPRT to obtain the ΔCt value. There was no detectable expression of IFN-α4 transcripts in uninfected control cells at timepoints past 6 hours or in the infected cells after day 3 post-infection. The shaded area indicates the range of IFN mRNA levels observed in uninfected, unstimulated controls over the experimental timecourse. The results are representative of findings made in 3 independent experiments conducted.

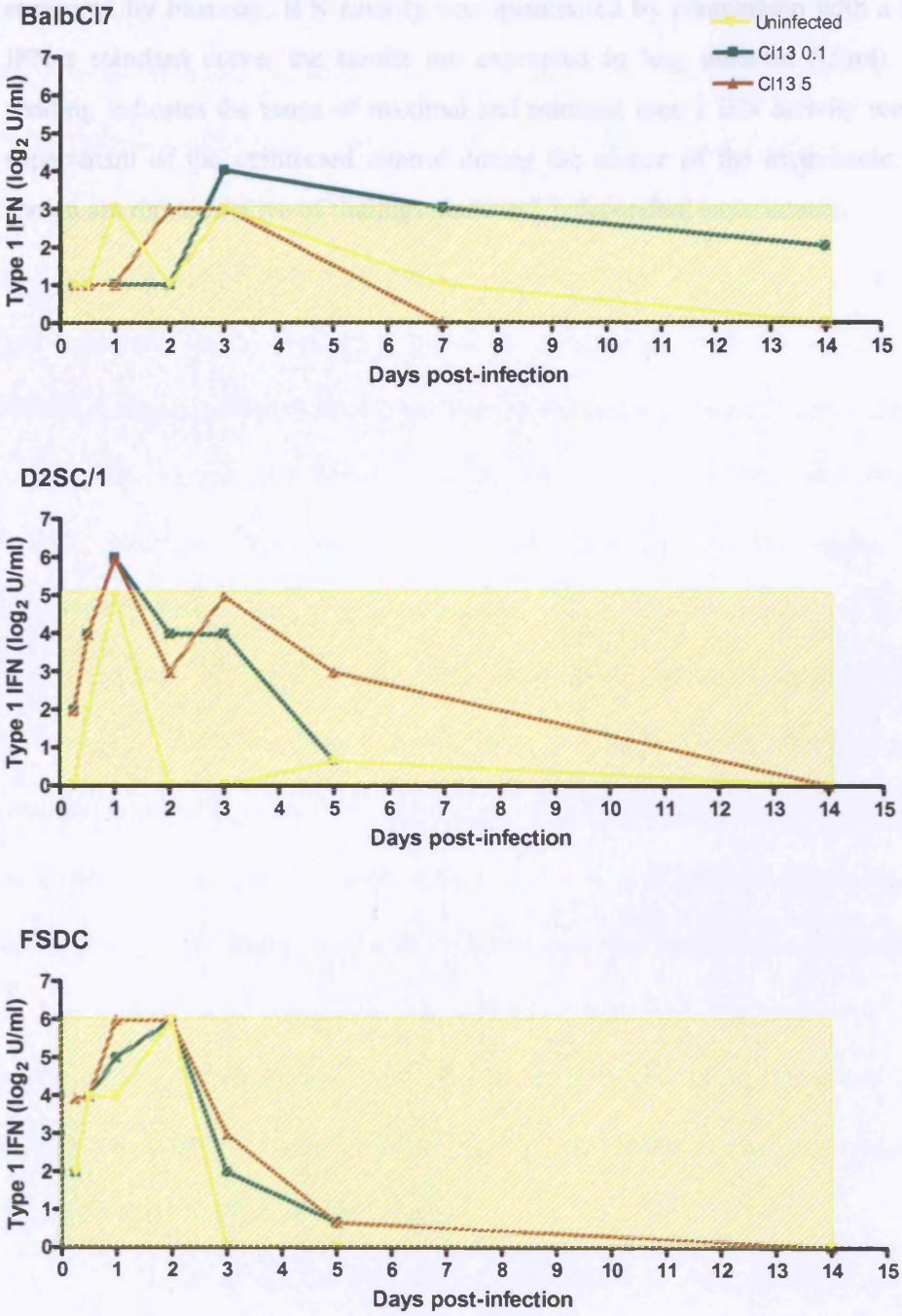

Figure 5.8 Type 1 IFN activity in the supernatant of BalbCl7, D2SC/1 or FSDC cells after infection at high or low m.o.i. with LCMV Cl13

A fibroblast cell line, BalbCl7, and two DC cell lines, FSDC and D2SC/1, were plated out at $1x10^6$ cells in a 1 ml volume into 60mm dishes or 6-well plates and were infected at low (0.1) or high (5/10) m.o.i. with LCMV Cl13. Supernatant was collected from individual wells at the timepoints indicated and the level of type 1 IFN activity was

measured by bioassay. IFN activity was quantitated by comparison with a recombinant IFN-α standard curve; the results are expressed in $\log_2$ units/ml (U/ml). The yellow shading indicates the range of maximal and minimal type 1 IFN activity recorded in the supernatant of the uninfected control during the course of the experiment. The results shown are representative of findings made in 3 independent experiments.

pathway(s) for type 1 IFN induction in uninfected cells.Uninfected BalbC17, D2SC/1 and FSDC cells were infected with other viruses, (the RNA virus, Sendai, and the DNA virus herpes simplex virus (HSV), both of which are reported to be strong inducers of type 1 IFN *in vitro*) at a high m.o.i. (10 and 5 respectively), or were stimulated with polyIC, and cellular RNA and culture supernatants were collected for quantitation of type 1 IFN mRNA/activity levels. Figure 5.9 shows the mean of the maximal levels of type 1 IFN mRNA subtypes produced during multiple experiments in BalbC17 and FSDC cells upon infection/stimulation with Sendai, HSV or polyIC together with the levels induced by LCMV infection. The levels shown are expressed as the mean ΔΔCt (ΔCt infected/stimulated – ΔCt uninfected control) values. The ΔΔCt value of each subtype in the sample was calculated by subtracting the maximal ΔCt value obtained in the infected or stimulated cells from the maximal level (i.e. highest ΔCt-value) of each subtype recorded in the unstimulated uninfected cells from the corresponding experiment.

In BalbC17 cells, infection with a high m.o.i. of LCMV stimulated a strong IFN-β response above the background and low to modest responses of the IFN-α subtypes. Even so, these levels were lower than the responses (especially the IFN-α response) induced upon infection with high doses (m.o.i. of 10 and 5 respectively) of Sendai or HSV. PolyIC stimulation of BalbC17 cells generated type 1 IFN responses that were broadly similar to the levels induced after LCMV infection.

In FSDC cells, infection with a high m.o.i. (10) of LCMV induced low to modest levels of IFN-β, IFN-α4 and other IFN-α subtypes above the background. In contrast, infection with high m.o.i. of Sendai and HSV generated much higher levels of all type 1 IFN subtypes measured, as compared to the levels induced upon infection with LCMV. Stimulation with polyIC induced responses of IFN-β, IFN-α4 that were largely equivalent to the levels

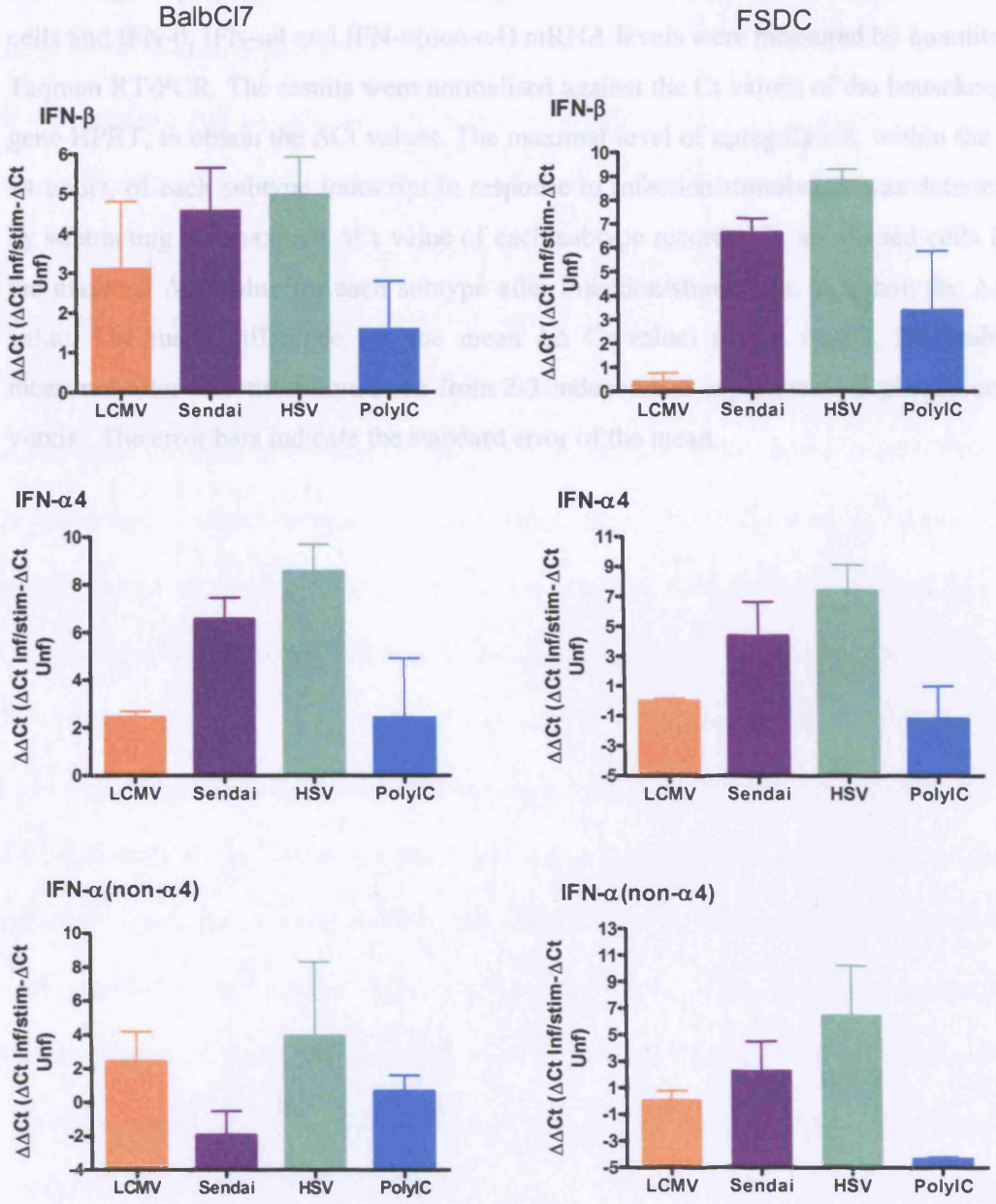

Figure 5.9 Maximal expression of IFN-β, IFN-α4 and IFN-α(non-α4) mRNA subtypes in BalbCl7 and FSDC cells in response to viral infection or polyIC stimulation

A fibroblast cell line, BalbCl7 and a DC cell lines FSDC were seeded into 6-well plates at $1x10^6$cells/well in 1 ml volume, or into 96-well plates at $1x10^5$cells/well in 100μl volume, and were infected with either LCMV Cl13 at a m.o.i. of 10, another RNA virus, Sendai, at a m.o.i. of 10; a DNA virus, HSV at a m.o.i. of 5 or were stimulated with 50μg/ml of the

TLR 3 ligand polyIC. At a series of timepoints thereafter, RNA was extracted from the cells and IFN-β, IFN-α4 and IFN-α(non-α4) mRNA levels were measured by quantitative Taqman RT-PCR. The results were normalised against the Ct values of the housekeeping gene HPRT, to obtain the ΔCt values. The maximal level of upregulation, within the first 24 hours, of each subtype transcript in response to infection/stimulation was determined by subtracting the maximal ΔCt value of each subtype recorded in uninfected cells from the maximal ΔCt value for each subtype after infection/stimulation to obtain the ΔΔ Ct value. The mean difference (i.e the mean ΔΔ Ct value) of the type 1 IFN subtype measured after infection/stimulation from 2-3 independent experiments is plotted on the y-axis. The error bars indicate the standard error of the mean.

induced upon infection with high m.o.i. of LCMV but very low levels of other IFN-α subtypes were upregulated.

It thus appears that BalbC17 and FSDC cells are able to strongly upregulate type 1 IFN when infected by RNA or DNA viruses. However, infection with high m.o.i. of LCMV did not induce as strong a type 1 IFN response in BalbC17 fibroblast and FSDC DC cells as generated during high dose infection of Sendai or HSV.

These results were reinforced by results from analysis of IFN activity levels in cell supernatant collected in parallel (Figure 5.10). In BalbC17 cells, infection with HSV and Sendai viruses induced secretion of high levels of type 1 IFN (~1024 or ~2^{10} U/ml), while polyIC did not induce significant type 1 IFN production, likely because of a lack of TLR3 expression in this fibroblast cell line. Infection with LCMV Cl13 did induce some type 1 IFN production, but the peak levels of IFN activity in the supernatant of LCMV-infected cells were much lower than those induced following infection with Sendai virus or HSV. The difference in the levels of type 1 IFN induced after LCMV versus HSV or Sendai infection was even more marked in the DC cell lines, where the peak levels in the supernatant of LCMV infected cells were not significantly higher than those present in the supernatant of uninfected cells. Unlike BalbC17 cells, the DC cell lines did also produce moderate amounts of type 1 IFN in response to stimulation with polyIC. Together, these results indicated that BalbC17, D2SC/1 and FSDC cells were capable of producing relatively high levels of type 1 IFN in response to infection with viruses including Sendai and HSV, suggesting that the comparatively weak type 1 IFN response observed following infection of these cells by LCMV may reflect a virus-induced block in type 1 IFN upregulation in infected cells.

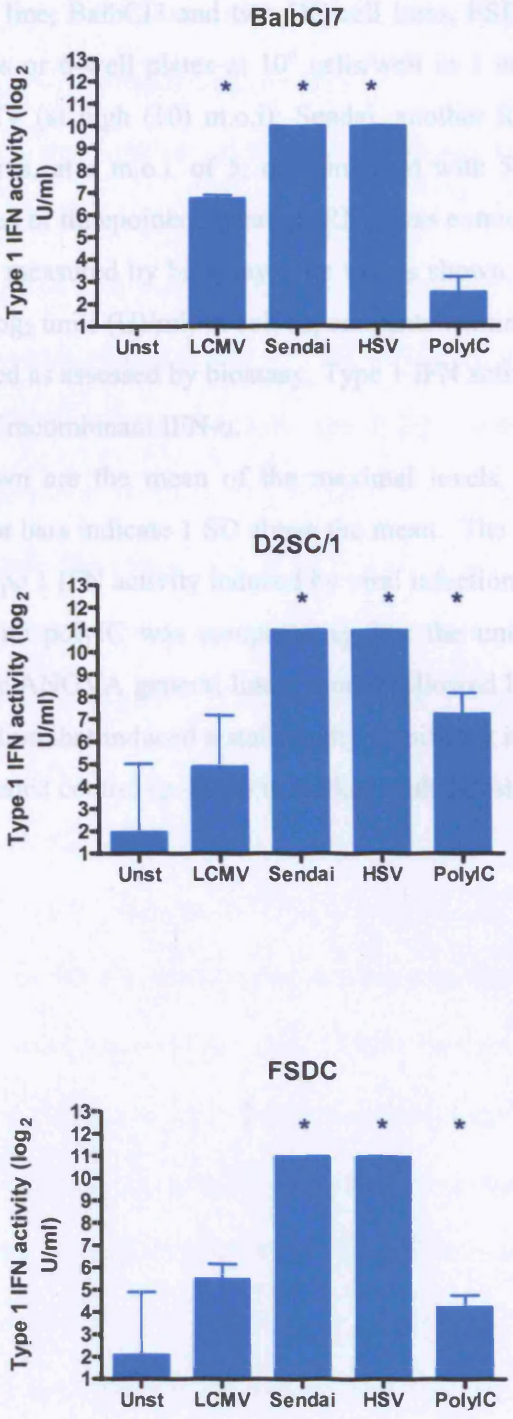

Figure 5.10 Maximal levels of supernatant type 1 IFN activity induced following stimulation of BalbCl7, D2SC/1 or FSDC cells with polyIC or infection with different viruses (figure legend on the following page)

A fibroblast cell line, BalbC17 and two DC cell lines, FSDC and D2SC/1 were seeded into 60mm dishes or 6-well plates at 10^6 cells/well in 1 ml volume, and were infected with either LCMV (at high (10) m.o.i); Sendai, another RNA virus, at a m.o.i. of 10; HSV, a DNA virus, at a m.o.i. of 5; or stimulated with 50ug/ml of the TLR 3 ligand polyIC. At a series of timepoints thereafter, RNA was extracted from the cells and type 1 IFN activity was measured by bioassay. The values shown are the peak levels of type 1 IFN activity (in $\log_2$ units (U)/ml) in cell supernatants within 24 hours after the cells were infected/stimulated as assessed by bioassay. Type 1 IFN activity was quantitated against a standard curve of recombinant IFN-α.

The results shown are the mean of the maximal levels from at least 2 independent experiments; error bars indicate 1 SD above the mean. The significance of the difference in the levels of type 1 IFN activity induced by viral infection with LCMV, Sendai or HSV or stimulation with polyIC was compared against the uninfected, unstimulated (Unst) baseline using the ANOVA general linear model followed by Tukey's simultaneous test. Infection/stimulation that induced a statistically significant increase in type 1 IFN activity over the unstimulated control ($p<0.05$) is marked with an asterisk (*).

5.5 Levels of type 1 IFN induced in LCMV-infected BalbCl7, D2SC/1 and FSDC cells after infection with Sendai virus or HSV

Further experiments were carried out to address whether cells infected with LCMV also were able to produce type 1 IFNs in response to infection with viruses shown in Figure 5.9/5.10 to elicit type 1 IFN production in uninfected cells. BalbCl7, D2SC/1 and FSDC cells which were persistently infected with LCMV were infected with a second virus, either Sendai or HSV and the levels of type 1 IFN induced by the second infection measured and compared to the levels secreted by previously uninfected cells upon infection with Sendai/HSV. Total cellular RNA and culture supernatants were collected at 4 and 8 hours post-infection and type 1 IFN mRNA levels and activity were quantitated as in previous experiments. Figure 5.11 shows the levels of the type 1 IFN in uninfected and persistently LCMV infected FSDC cells 4 hours after infection with Sendai virus or HSV. As previously observed, uninfected cells responded to infection with of Sendai virus or HSV by upregulating expression of IFN-β, IFN-α4 and other IFN-α subtypes, with infection by HSV eliciting a stronger response than infection with high or low m.o.i. of Sendai. In cells persistently-infected with LCMV, infection with HSV also induced a strong upregulation of all type 1 IFN subtypes, although the levels produced were slightly lower than those made by similarly exposed uninfected cells. However, when persistently-infected cells were infected with Sendai virus at low or high m.o.i., although there was some upregulation of all subtypes of type 1 IFN, the levels produced were significantly lower at 18 hours post-infection than those made by Sendai-infected control cells. In line with this, levels of Mx mRNA were upregulated in both uninfected and persistently infected cells upon infection with HSV. Uninfected cells also upregulated Mx when infected with low or high doses of Sendai virus Mx mRNA levels in persistently infected cells did not increase when these cells were infected with Sendai virus.

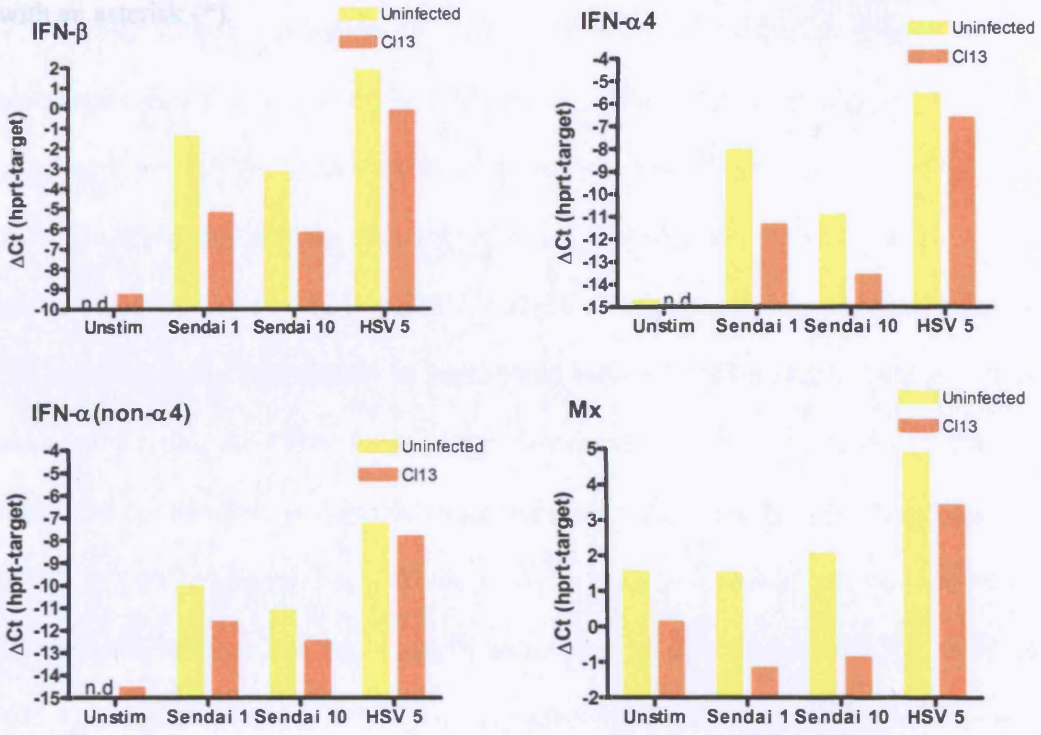

Figure 5.11 Type 1 IFN and Mx mRNA upregulation in FSDC cells persistently infected with LCMV in response to Sendai or HSV infection

Uninfected FSDC cells and FSDC cells infected >80 days previously with LCMV Cl13 were infected with Sendai virus at a m.o.i of 1 or 10 or with herpes simplex virus (HSV) at a m.o.i of 5. Control cells were LCMV Cl13-infected or uninfected cells that were not super-infected with any other virus (unstim). At 4 hours post-infection, cells were lysed in RLT buffer for mRNA isolation and type 1 IFN/Mx mRNA levels were determined by quantitative Taqman RT-PCR. The Ct value of each type 1 IFN/Mx gene was normalised by subtraction from the Ct value of the housekeeping gene HPRT; the normalised level for each gene is displayed as a ΔCt value while n.d. indicates target mRNA was not detected in the sample. The results shown are representative of observations made in 2 independent experiments with FSDC cells and a further 2 independent experiments conducted on BalbCl7 cells. The difference in the levels of type 1 IFN mRNA subtype induced by Sendai or HSV infection in uninfected or cells persistently infected with LCMV Cl13 was compared using the ANOVA general linear model followed by Tukey's simultaneous test. Infections that induced a statistically significant difference ($p<0.05$) in

the level of type 1 IFN subtype in uninfected versus persistently infected cells are marked with an asterisk (*).

These observations at the transcript level were backed up by results of analysis of type 1 IFN activity in the supernatant of cells at 18 hours post-infection (Figure 5.12). In uninfected cells, high levels of type 1 IFN activity were produced in response to infection with Sendai or HSV (~2^{11} U/ml), indicating that both viruses stimulated strong type 1 IFN responses. However, whereas the levels of type 1 IFN secreted by uninfected and LCMV-infected cells upon infection with HSV, a DNA virus, were similar, the levels of type 1 IFN detected in the supernatant of persistently infected cells infected with low or high doses of Sendai, an RNA virus, were significantly lower than those produced by uninfected control cells in response to infection with this virus ($p=0.0013$, as determined by the ANOVA general linear model). These results indicated that the persistently infected cells had not lost their capacity to respond to an infection with the DNA virus HSV by production of type 1 IFN, but suggested that their ability to respond to infection by an RNA virus, Sendai may be impaired. Similar observations were also made in experiments carried out using the fibroblast cell line BalbC17 (data not shown).

To exclude the possibility that the persistently infected cells had a pre-existing antiviral state and so were resistant to a further infection with Sendai, real-time Taqman PCR assays were used to quantify Sendai virus RNA NP transcripts in uninfected and persistently-infected cells at different timepoints following infection with Sendai virus. The results obtained (Figure 5.13) indicated that both uninfected and LCMV-infected cells were infected equally well by Sendai virus. Equivalent levels of Sendai virus NP RNA were detected in both cell populations at 4 hours post-infection and had increased to a similar extent by 18 hours post-infection, indicative of viral replication occurring in a parallel fashion in both control and LCMV Cl13-infected cells.

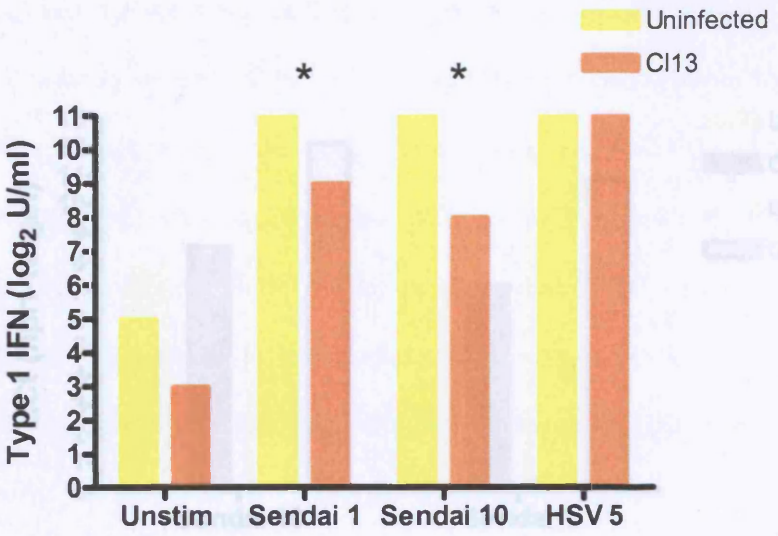

Figure 5.12 Type 1 IFN activity detected in the supernatant of uninfected FSDC and FSDC cells persistently infected with LCMV following infection with Sendai virus or HSV

FSDC cells infected >80 days previously with LCMV Cl13 were infected with Sendai virus at a m.o.i. of 1 or 10 or herpes simplex virus (HSV) at an m.o.i. of 5. Control cells were LCMV Cl13 infected or uninfected cells that were not super-infected with any other virus (unstim). At 18 hours post-infection, supernatants were collected and the level of type 1 IFN activity they contained was measured by antiviral EMCV bioassay. The results are expressed as $\log_2$ units of IFN activity per ml ($\log_2$ U/ml). The results shown are representative of observations made in 2 independent experiments with FSDC cells and a further 2 independent experiments conducted on BalbC17 cells. Statistical analysis using ANOVA general linear model to compare the levels of type 1 IFN induced after uninfected or persistently infected cells were exposed to Sendai or HSV suggested that there was a statistically significant difference in the level of type 1 IFN produced by uninfected and persistently infected cells following infection with a low or high m.o.i. of Sendai virus ($p<0.05$), indicated by an asterisk (*), but not after infection with HSV.

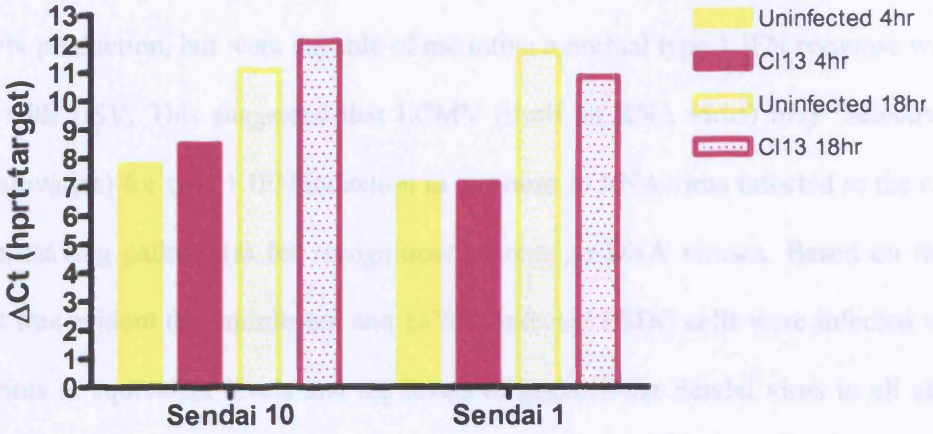

Figure 5.13 Sendai virus RNA levels in control and LCMV Cl13-persistently infected FSDC cells 4 and 18 hours after infection with Sendai virus

Uninfected FSDC cells and FSDC cells infected >80 days previously with LCMV Cl13 were infected with Sendai virus at a m.o.i. of 1 or 10. Total RNA was extracted at 4 and 18 hours post-Sendai infection and Sendai virus mRNA levels were determined by quantitative Taqman RT-PCR. The level of Sendai viral transcript detected in each sample was normalized against the housekeeping gene HPRT which was analysed in parallel, and the values are expressed as ΔCt. The results shown are representative of findings made in two independent experiments.

Together, there results indicated that cells persistently-infected with LCMV Cl13 had an impaired capacity to respond to infection with Sendai virus protein by upregulation of type 1 IFN production, but were capable of mounting a normal type 1 IFN response when infected with HSV. This suggested that LCMV (itself an RNA virus) may selectively impair pathway(s) for type 1 IFN induction in response to RNA virus infected in the cells it infects, leaving pathway(s) for recognition/response to DNA viruses. Based on these assays, it was evident that uninfected and LCMV-infected FSDC cells were infected with Sendai virus at equivalent levels and the levels of intracellular Sendai virus in all cases increased at 18 hours post-infection which was indicative of viral entry and replication, leaving pathways for recognition of/response to DNA virus infection intact.

5.6 Type 1 IFN activity in the supernatant of persistently infected cells after stimulation with synthetic TLR ligands

There are several ways in which cells can senses the presence of viral RNA, including through TLR3 or TLR7, which recognize double stranded or single stranded RNA, respectively, and via a cytoplasmic pathway initiated by activation of helicases such as RIG-1 or MDA5 (Kato et al. 2006). To assess the ability of cells persistently infected with LCMV Cl13 to respond to TLR stimuli, infected cells were stimulated with polyIC, R848 or CpG1826 and the level of type 1 IFN production was analysed. Initial experiments, the results of which are shown in Figure 5.14 revealed that when uninfected, the DC lines were responsive to polyIC, LPS, R848 and CpG, producing moderate to high levels of type 1 IFN in response to all these stimuli. As can be seen in Figure 5.15, there was no difference in the level of type 1 IFN induced by the synthetic TLR ligands in uninfected and persistently infected DC. The data in Figure 5.15 show that the TLR 3, 7 and 9 stimulated pathways for type 1 IFN induction were not impaired in cells persistently

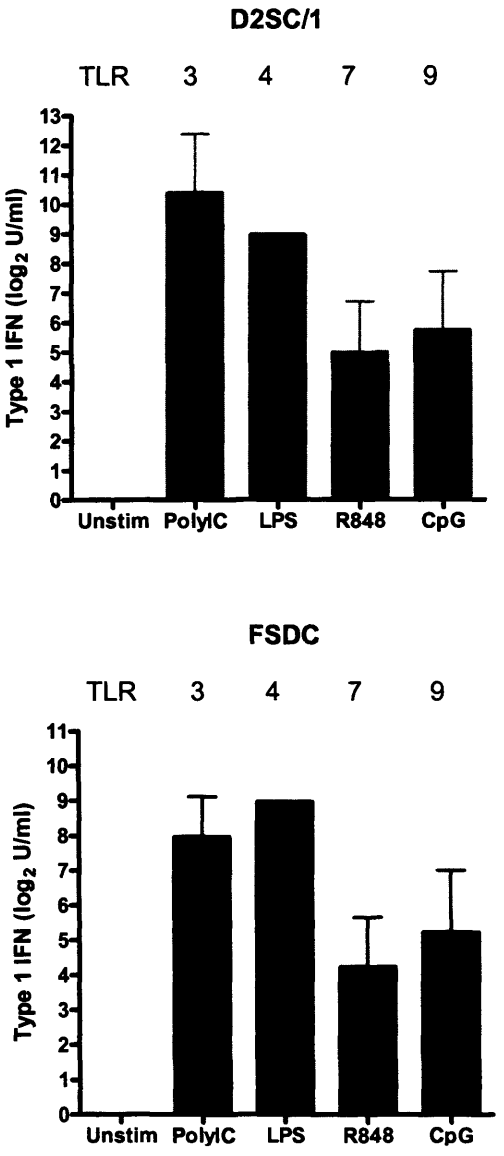

Figure 5.14 Type 1 IFN production by D2SC/1 and FSDC cells in response to stimulation with different TLR ligands

Two DC cell lines, D2SC/1 and FSDC, were seeded into 96-well plates at $1x10^5$ cells/well in 100μl of media and stimulated with polyIC at 100μg/ml, LPS at 10μg/ml, R848 at 10μg/ml or CpG 1826 at 0.2nmol/ml (the TLR stimulated by each ligand is indicated). Supernatants were harvested the following day and the levels of type 1 IFN produced evaluated by bioassay. A standard curve of recombinant IFN-α was set up in parallel. The results shown are the levels of type 1 IFN, expressed as log_2 units/ml (U/ml).

The results are the mean of values obtained from 2-4 independent experiments, with one standard error above the mean depicted by the error bars.

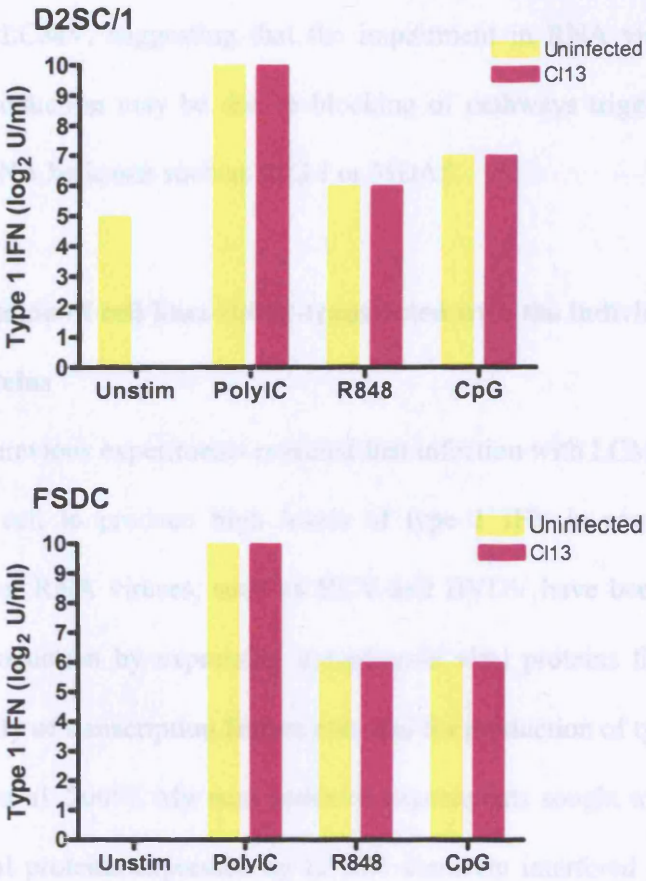

Figure 5.15 Type 1 IFN production in response to TLR stimuli by DC cell lines persistently infected with LCMV

D2SC/1 or FSDC cells infected >30 days previously with LCMV Cl13 were plated out at $1x10^5$ cells/well into 24-well plates and stimulated overnight with the following TLR ligands: polyIC (100µg/ml), R848 (50µg/ml) or CpG 2216 (2nmol/ml). The supernatant was collected and type 1 IFN activity was measured by bioassay. A standard curve of two-fold serial dilutions of recombinant IFN-α was set up in parallel to quantitate type 1 IFN activity in the samples. The results are expressed as log_2 units/ml (U/ml) of type 1 IFN activity and are representative of findings made in two independent experiments. Statistical analysis using ANOVA general linear model suggests that there was no significant difference in the level of type 1 IFN activity induced by the various TLR ligands in uninfected and LCMV Cl13 persistently-infected cells ($p>0.05$).

infected with LCMV, suggesting that the impairment in RNA virus infection-induced type 1 IFN production may be due to blocking of pathways triggered by activation of cytoplasmic RNA helicases such as RIG-I or MDA5.

5.7 Generation of cell lines stably-transfected with the individual LCMV GP, NP or Z viral proteins

The results of previous experiments revealed that infection with LCMV interferes with the ability of the cell to produce high levels of type 1 IFN in response to RNA virus infections. Other RNA viruses, such as HCV and BVDV have been reported to impair type 1 IFN production by expressing cytoplasmic viral proteins that interact with and block the activity of transcription factors essential for production of type 1 IFN (Baigent et al. 2002; Foy et al. 2005). My next series of experiments sought to investigate whether one of the viral proteins expressed by LCMV similarly interfered with the type 1 IFN induction pathway to block or reduce type 1 IFN production. DC cell lines were stably transfected with plasmids encoding individual LCMV proteins, and the effects of expression of each protein on the ability of the cells to produce type 1 IFNs in response to infection with Sendai virus was investigated.

Plasmids containing single genes of the LCMV genome, encoding the glycoprotein GP, nucleoprotein NP or the Z protein respectively, were transfected into FSDC or D2SC/1 cells. The cells, in 6-well plates, were co-transfected with the LCMV plasmid and the plasmid pcDNA-V5-HisB which encodes a protein that confers resistance to blasticidin. The LCMV and pcDNA-V5-HisB plasmids were mixed at a ratio of 5:1 prior to transfection in order to increase the chance that a cell that internalized the blasticidin resistance plasmid would also co-internalize the LCMV plasmid. Transfected cells were then grown in media containing blasticidin in order to select for cells which had

successfully internalized and expressed the plasmids. The colonies of blasticidin-resistant cells were pooled, and plated out into 96-well plates using limiting dilution at a concentration of 1 cell per well in order to generate single-celled clones of the transfected cells. Following expansion, selected clones co-transfected with each of the LCMV plasmids were screened for expression of the LCMV protein in the cells, which were confirmed by immunofluorescent staining. For cells transfected with the GP, GP expression was detected using the anti-LCMV ascitic fluid VR134, followed by an anti-mouse Ig-FITC conjugate. Expression of the LCMV NP protein in the NP-plasmid transfected clones was detected using the anti-NP-specific monoclonal antibody (mAb) NP-113 conjugated to Cy5. Unfortunately, no antibodies were available for detection of the LCMV Z protein. However, given that the high proportion of the GP- and NP-transfected clones that were found to express the relevant proteins, (9/10 GP-transfected clones and 9/11 NP-transfected clones), made it worthwhile to proceed with further experiments on a randomly selected Z-transfected clone to test in parallel with the GP- and NP-expressing clones. One clone from each transfected plasmid was selected and expanded in blasticidin-supplemented growth medium. Prior to experimental use, the selected clones were tested again by immunofluorescent staining to confirm that they were still expressing the relevant viral proteins. Representative examples of confocal microscopy images from an immunoflurescence assay conducted on the GP and NP-expressing cell lines are shown in Figure 5.16. No fluorescence was observed when cells transfected with the blasticidin-resistence plasmid only were stained with VR-134 ascitic fluid whereas cells transfected with the LCMV GP fluoresced. Further, when cell lines transfected with the LCMV NP and GP were stained with the mAb NP113, fluorescent cells were only observed in the cell line expressing the NP protein, and not GP.

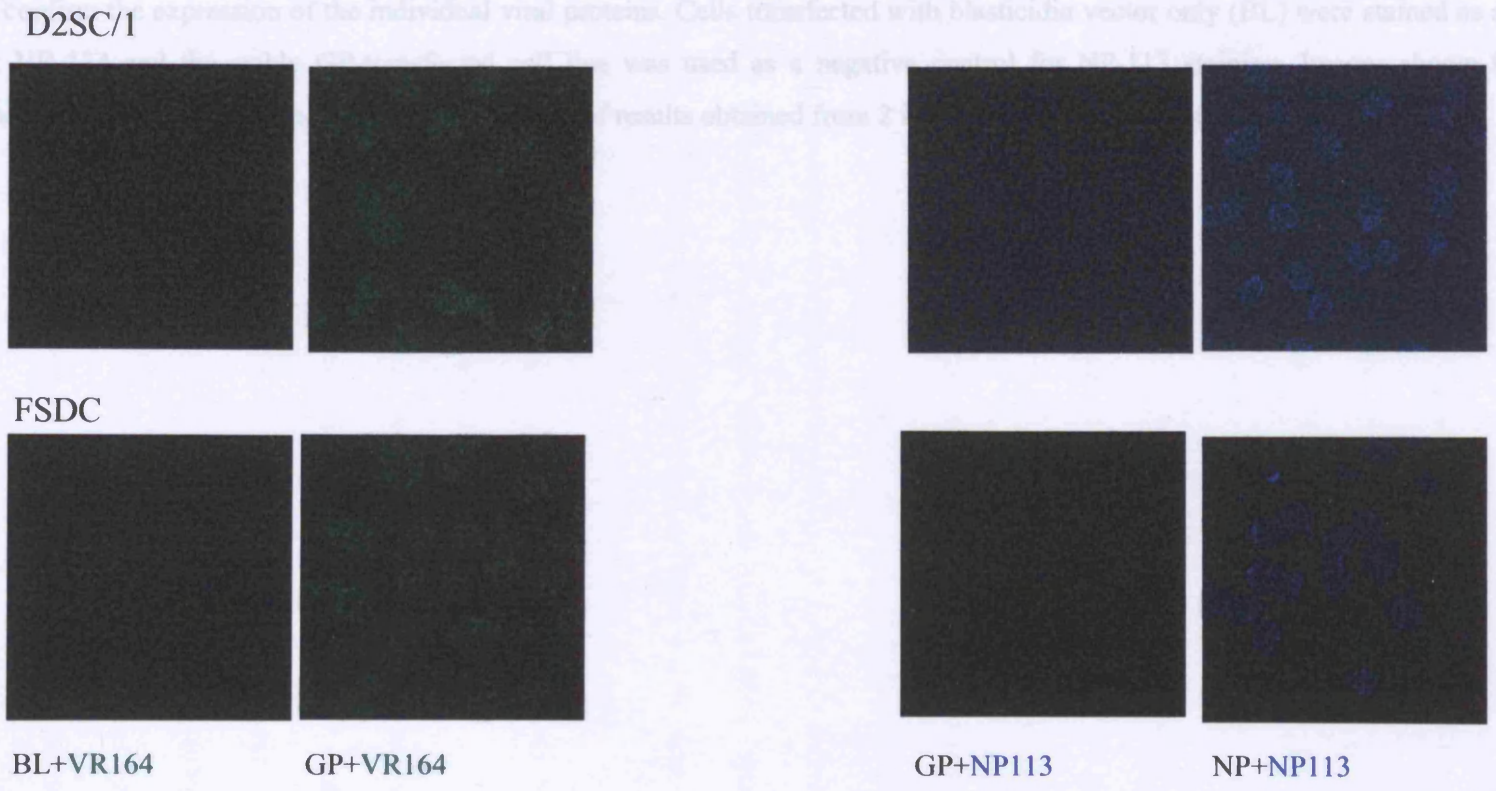

Figure 5.16 Staining of transfected D2SC/1 and FSDC to verify expression of the LCMV GP or NP

D2SC/1 and FSDC cells which were stably transfected with expression vectors encoding the GP or NP proteins of LCMV were stained with either VR-134, followed by anti-mouse Ig-FITC (green) to detect the GP proteins or NP113-Cy5 mAb (blue) which recognized the NP protein,

in order to confirm the expression of the individual viral proteins. Cells transfected with blasticidin vector only (BL) were stained as a negative control for VR-134 and the stably GP-transfected cell line was used as a negative control for NP-113 staining. Images shown here were photographed at 100x magnification and are representative of results obtained from 2 independent experiments.

5.8 Type 1 IFN production by stably-transfected cells in response to infection with Sendai virus or HSV

Untransfected cells and cell lines stably transfected with the blasticidin resistance plasmid only or this plasmid and LCMV GP, NP or Z-encoding plasmids were plated into 96-well plates and infected with Sendai virus at a m.o.i. of 1 or 10 or HSV at a m.o.i. of 5, and the type 1 IFN response induced in was measured. The levels of type 1 IFN mRNA induced in the uninfected and transfected cells were measured by real-time quantitative RT-PCR and the results are shown in Figure 5.17. As expected, type 1 IFN mRNA was upregulated in both cell lines when untransfected, after infection with both viruses. Notably, the level of type 1 IFN mRNA induced following infection in cell lines expressing individual LCMV proteins was not any lower than that induced in the untransfected cells or cells transfected with the blasticidin resistance plasmid only in response to infection. Bioassay analysis of type 1 IFN activity in culture supernatants, as shown in Figure 5.18, revealed similar results, i.e. all the infected cells produced type 1 IFN after infection, there was no difference in the levels secreted by untransfected cells, cells transfected with the blasticidin-resistance plasmid only or any of the cell lines transfected with plasmids encoding individual LCMV proteins.

These preliminary experiments do not provide any evidence to suggest that individual LCMV proteins impair intracellular pathways involved in upregulation of type 1 IFN production in response to RNA virus infection. However, these results must be interpreted with caution. As explained above, it was not possible to verify expression of the LCMV Z protein in transfected cells – hence this protein may not have been produced. Despite the fact that GP and NP were shown to be expressed in transfected cells, it is possible that the levels of protein produced, their intracellular location and/or their processing may have differed from that in LCMV-infected cells.

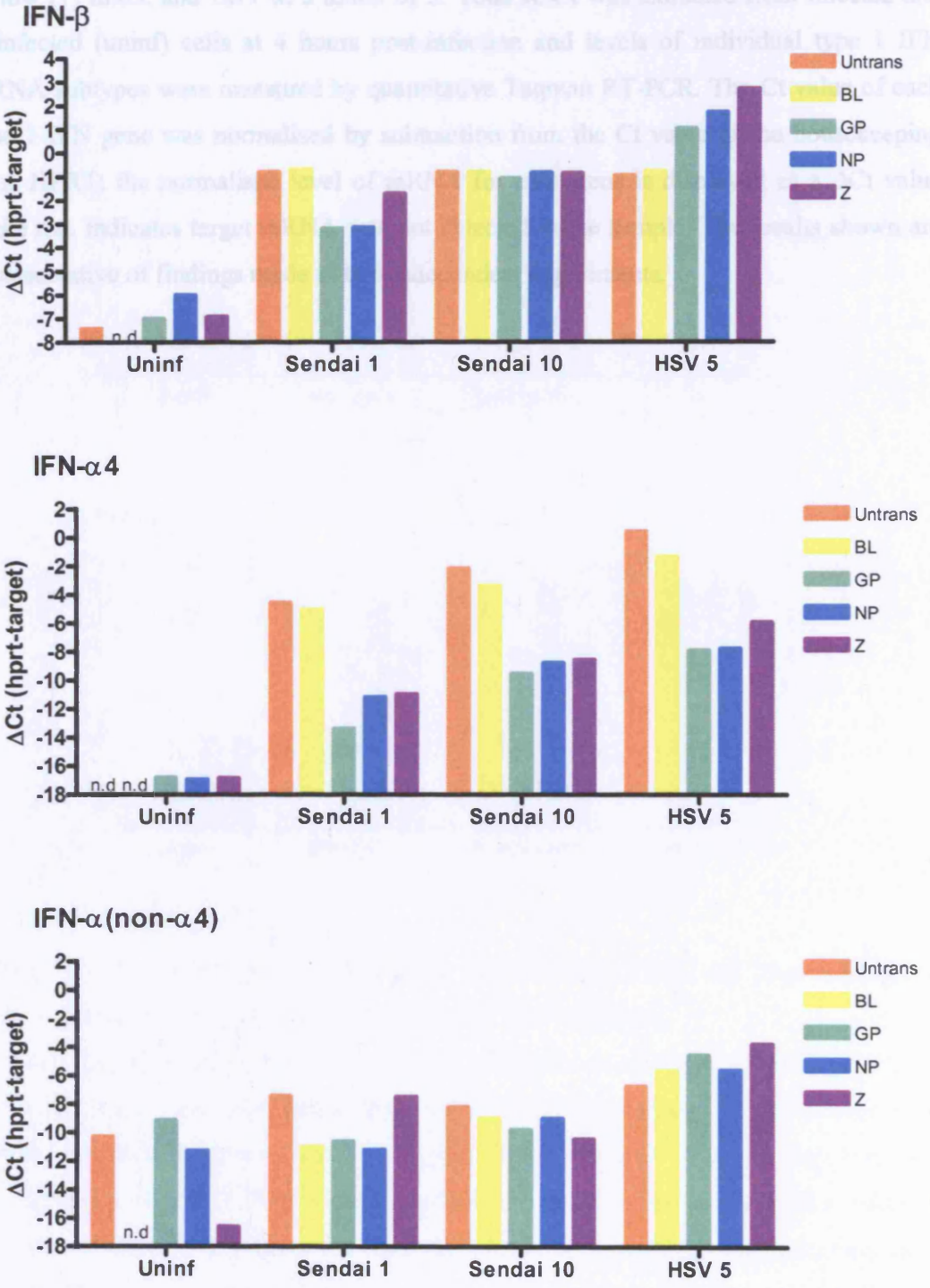

Figure 5.17 Levels of type 1 IFN mRNA induced in LCMV-protein-transfected FSDC cells upon infection with Sendai virus or HSV

FSDC cells stably transfected with LCMV GP, NP or Z proteins or an empty plasmid (BL) and untransfected FSDC cells (untrans) were infected with Sendai virus at high (10)

or low (1) m.o.i. and HSV at a m.o.i. of 5. Total RNA was extracted from infected and uninfected (uninf) cells at 4 hours post-infection and levels of individual type 1 IFN mRNA subtypes were measured by quantitative Taqman RT-PCR. The Ct value of each type 1 IFN gene was normalised by subtraction from the Ct value of the housekeeping gene HPRT; the normalised level of mRNA for each gene is displayed as a ΔCt value while n.d. indicates target mRNA was not detected in the sample. The results shown are representative of findings made in two independent experiments.

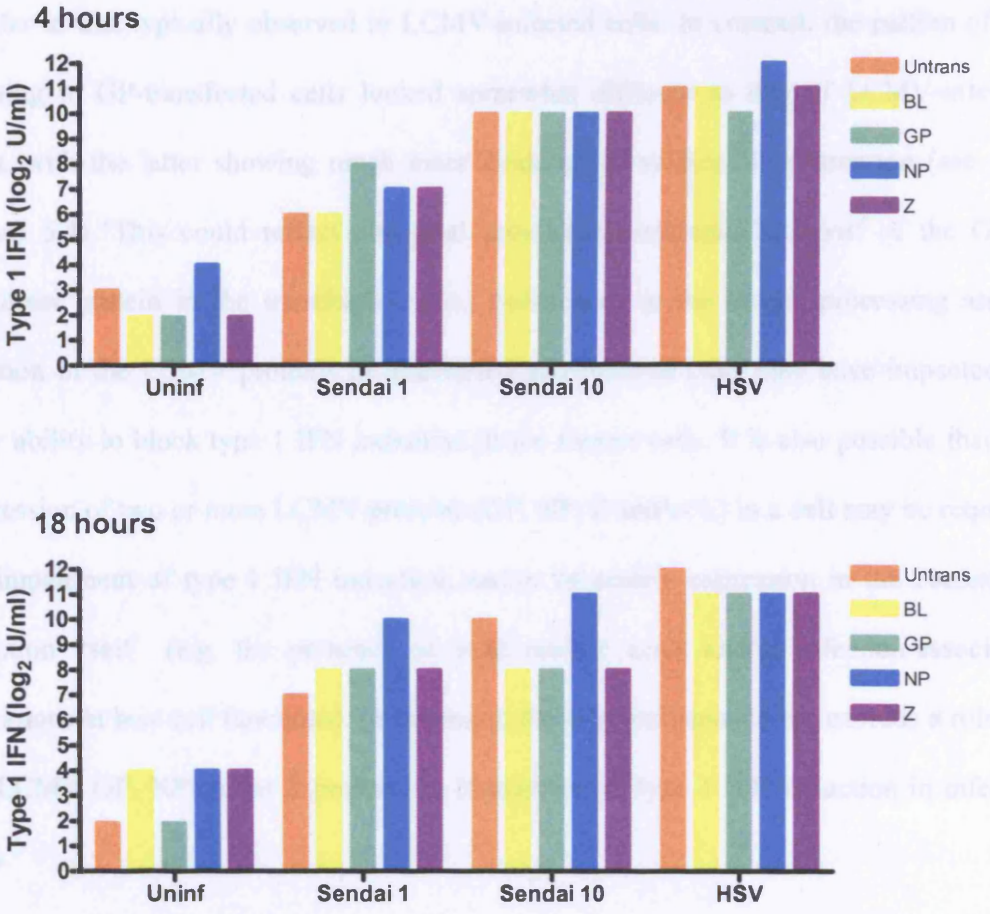

Figure 5.18 Levels of type 1 IFN activity produced by FSDC cell lines expressing LCMV proteins in response to Sendai virus or HSV infection

Plasmids encoding the GP, NP or Z proteins of LCMV or an empty plasmid (BL) were stably transfected into FSDC cells. These cell lines and untransfected (Untrans) cells were infected with Sendai virus at low (1) or high (10) m.o.i. or with HSV at a high m.o.i. (5) and the levels of type 1 IFN produced and released into the supernatant after infection were measured by EMCV bioassay. A standard curve of recombinant IFN-α was set up in parallel. The results shown are the levels of type 1 IFN, expressed as $\log_2$ units/ml (U/ml), present in the supernatant at 4 and 18 hours after infection and are representative of results obtained in 2 independent experiments.

The level and pattern of NP staining in NP-transfected cells (Figure 5.16) appeared fairly similar to that typically observed in LCMV-infected cells. In contrast, the pattern of GP staining in GP-transfected cells looked somewhat different to that of LCMV-infected cells, with the latter showing much more evidence of surface GP-expression (see e.g., Figure 5.4). This could reflect abnormal processing/maturation/transport of the GP-C precursor protein in the transfected cells. Differences in the levels, processing and/or location of the LCMV proteins in transfected and infected cells may have impacted on their ability to block type 1 IFN induction in the former cells. It is also possible that co-expression of two or more LCMV proteins (GP, NP, Z and/or L) in a cell may be required for impairment of type 1 IFN induction, and/or of protein expression in the context of infection itself (e.g. the presence of viral nucleic acids and/or infection-associated alterations in host cell functions). In summary, these experiments do not exclude a role for the LCMV GP, NP and/or Z proteins in impairment of type 1 IFN induction in infected cells.

5.9 Summary and Conclusion

LCMV has been reported to be able to infect most mammalian cell types. In agreement with this I found that LCMV Cl13 was able to infect bone-marrow derived DCs, the fibroblasts cell line BalbCl7 and the DC cell lines D2SC/1 and FSDC, establishing a productive infection with release of infectious progeny virus into the supernatant in each case. In the immortalized cell lines BalbCl7, D2SC/1 and FSDC, the infection persisted long-term, with release of infectious virus being observed for at least 27 days post-infection and the presence of LCMV proteins in the cells being demonstrated by immunofluorescent staining at >80 days post-infection.

In BM-DCs, infection with LCMV Arm or LCMV Cl13 induced upregulation of IFN-α4, IFN-β and other IFN-α subtype mRNA within the first 6 hours of infection. IFN-α4 and IFN-β mRNA continued to be expressed above baseline levels for up to 48 hour post infection. Some increase was also observed in type 1 IFN activity in the supernatant during the first 24 hours post-infection. However, the upregulation of type 1 IFN production observed was very modest when compared to the levels induced by the synthetic TLR3 ligand polyIC which stimulated much higher and more sustained upregulation of type 1 IFN mRNA and activity.

In line with this observation, LCMV infection of immortalized fibroblast and dendritic cell lines also induced very little upregulation of type 1 IFN production, with type 1 IFN mRNA levels in cells and activity in the supernatant being scarcely elevated above the baseline levels observed in uninfected cells throughout the experiment. Extensive fluctuation of type 1 IFN levels was observed in uninfected cells as they were cultured. Nonetheless, when compared to the levels of type 1 IFN induced in similar cell lines after infection with other viruses, LCMV still appeared to be a poor stimulator of type 1 IFN induction.

In order to determine if pathway(s) for type 1 IFN induction were impaired in LCMV infection-infected cells, immortalized cells persistently infected with LCMV Cl13 were infected with a second virus, either the RNA virus Sendai or the DNA virus HSV and the type 1 IFN response induced was evaluated. The results obtained suggested that there was impairment in type 1 IFN production in response to RNA virus infection in the persistently infected cells as lower levels of type 1 IFN were produced after Sendai virus infection of LCMV-infected cells than were produced after Sendai virus infection of uninfected cells, despite the fact that Sendai virus appeared to infect and replicate in both cell types equally well (as judged by analysis of Sendai virus RNA transcript levels over

time). By contrast, a similar impairment was not observed in the ability of persistently-infected cells to produce type 1 IFN in response to infection with HSV, suggesting that there may be a specific impairment of pathways involved in the recognition of and/or response to RNA virus infection in LCMV-infected cells.

When persistently-infected cells were stimulated with synthetic TLR stimuli polyIC, CpG1826 DNA or R848, there was no difference in the level of type 1 IFN activity induced in the LCMV-infected and uninfected control cells, indicating that the TLR-triggered pathways for type 1 IFN induction were still intact in the LCMV infected cells. This suggested that the impairment in the ability of LCMV-infected cells to produce type 1 IFN in response to RNA virus infection may be due to blocking of pathways triggered by activation of cytoplasmic helicases such as RIG-I and MDA5. To gain insight into whether one of the LCMV proteins was responsible for the partial impairment of type 1 IFN induction, cell lines stably transfected with plasmids encoding individual LCMV proteins (GP, NP or Z) were generated and their ability to upregulate type 1 IFN production in response to infection with Sendai virus was investigated. These cell lines were not found to exhibit a defect in production following infection with Sendai virus. These results did not provide evidence to support a role for the isolated, individual LCMV GP, NP or Z proteins in impairment of type 1 IFN induction in LCMV-infected cells; however further work would be required before such a role could be excluded.

CHAPTER 6

DISCUSSION

Available evidence suggests that most acute viral infections trigger a rapid and short-lived type 1 IFN response. Notably, this often starts to be downregulated before viral titres in the host have reached their peak (Biron and Sen 2001). Further, as reviewed in the introduction, persistent viral infections where there are high levels of ongoing viral replication are typically not observed to be associated with long term high level upregulation of type 1 IFN production. Instead, there may be low-level type 1 IFN production, a lack of type 1 IFN upregulation, or suppression of type 1 IFN induction (Anthony et al. 2004; Duan et al. 2004).

A number of mechanisms may contribute to downregulation of the acute-phase type 1 IFN response, and/or suppression of type 1 IFN production in the context of chronic viral replication. Many viruses have evolved strategies for impairing pathways involved in upregulation of expression of type 1 IFNs (and/or IFN-stimulated viral genes) as a means to prolong their survival in the host. As reviewed in the introduction (see Figure 1.1 and 1.2), type 1 IFN may be blocked at the level of pathogen component recognition, by sequesteration of ligands which may trigger type 1 IFN induction, e.g. dsRNA (Talon et al. 2000); the signaling pathway involved in IFN gene regulation may be targeted, e.g. HCV impairs the MAVS adaptor protein (Foy et al. 2005); type 1 IFN binding to its receptor may be impaired or signaling through the IFN-R may be affected, e.g. through interference with the JAK/STAT signaling pathway (Didcock et al. 1999). Restriction of type 1 IFN production in acute or chronic viral infections may thus reflect a virus-induced block.

Virus infections may also reduce type 1 IFN production via detrimental effects on the generation, survival and/or functions of key IFN-producing cells such as plasmacytoid

DCs. Viruses that infect plasmacytoid DCs (e.g. HIV-1) may have direct effects on the survival/functions of this important IFN-producing cell type (Flano et al. 2005; Pollara et al. 2005). However, virus infections may also impact on plasmacytoid DC numbers more indirectly, via effects on pathways controlling their generation/survival, e.g. persistent expression of type 1 IFN during chronic LCMV Cl13 infection has been reported to suppress the maturation of DC precursors in the spleen (Hahm et al. 2005).

In addition, the host possesses homeostatic mechanisms for downregulation of type 1 IFN production, because inappropriate expression of type 1 IFN may have pathogenic effects e.g. some of the pathology observed in mice neonatally challenged with LCMV is reported to result from the toxic effects of excessive type 1 IFN induced in response to LCMV infection (Gresser et al. 1978; Riviere et al. 1977). As discussed in the introduction, expression of type 1 IFN is typically followed by appropriate activation of ISGs as well as repression of further IFN activity and expression, e.g by upregulation of IRF-4 or receptor internalization and degradation. However, little is known as to whether these homeostatic mechanisms continue operating during long-term productive viral infections.

In summary, the relative contribution of different mechanisms to the regulation of type 1 IFN production in acute and persistent viral infections remains poorly understood.

In this project, I aimed to characterize the type 1 IFN response in the acute and chronic phases of infection of mice with a natural murine pathogen, LCMV, and to analyse the ability of the virus to modulate type 1 IFN production in the cells it infects.

LCMV isolates differ in their ability to establish persistent infections in adult immunocompetent C57BL/6 mice, e.g. LCMV Arm typically causes an acute infection which is controlled in 7-14 days while the LCMV Docile and LCMV Cl13 causes a persistent infection if it is delivered systemically, via the i.v. route at high doses (e.g. of about 10^6 p.f.u). Infection is non-cytopathic; and the virus quickly replicates to high viral titres in many organs and infectious virus is secreted for extended periods (Borrow and Oldstone 1997). Initial experiments tested strains of LCMV available in the laboratory to determine whether they were able to cause a long-lived infection (>4 weeks) in adult immunocompetant C57BL/6 mice. While two of the LCMV isolates available to me, namely LCMV Cl13 and LCMV Docile, had been previously reported to establish persistent infections in wild-type adult C57BL/6 mice, the working stocks employed here had been passaged several times in tissue culture, which may have affected the *in vivo* virulence of the strains. A pilot experiment was thus conducted to determine how long the newly-generated working stocks persisted *in vivo*. Future experiments aimed to investigate the innate immune response in a background of persistent LCMV infection, and so it was imperative that the mice remained infected for longer than 3 weeks in order to allow "exhaustion" of the adaptive T-cell response to occur and chronic infection to be established.

LCMV Cl13 and LCMV Docile were both found to persist in wild-type adult C57BL/6 mice for a period of >4 weeks; however LCMV Cl13 was eventually selected for experimental use because it possessed a sequence that was almost identical to a LCMV strain available in the laboratory, which causes an acute infection, namely LCMV Arm. The genome of both LCMV Arm and LCMV Cl13 have been sequenced and found to differ by only 2 amino acids, resulting in a phenylalanine to leucine substitution at position 260 in GP-1 and lysine to glutamine

at position 1079 in the viral polymerase of LCMV Cl13 (Matloubian et al. 1993; Matloubian et al. 1990; Salvato et al. 1988)(Salvato et al Virology, 1988 164, 517). The use of closely-matched viral isolates is preferable as it facilitates comparative analysis of results.

Experiments were then conducted to follow the type 1 IFN responses in mice infected with LCMV Arm or LCMV Cl13. Type 1 IFN levels were assessed by measurement of transcript abundance in the spleen and anti-viral activity in the serum. Both methods indicated that in mice infected with LCMV Arm, which causes an acute infection, type 1 IFN production reached maximal levels by 12-24 hours post-infection. Upregulation of IFN-β, IFN-α4 and IFN-α(non-α4) mRNA and activity reached maximal levels within 24 hours after LCMV infection, and then fell by 72 hours post-infection before dropping to baseline/undetectable levels between days 7 to 21 when the infection was resolved. Thus, during acute infection, type 1 IFN induction in the spleen and blood was transient and occurred very quickly post-infection. Viral plaque assays performed on the same samples show that in mice infected with LCMV Arm, the type 1 IFN response declined before viral replication reached its peak, which occurred at ~72 hours post-infection. Investigations in mice infected with LCMV Cl13, which is able to establish a persistent infection showed that type 1 IFN was induced with similar kinetics of induction after infection with LCMV Arm. The magnitude of the peak type 1 IFN response induced in the spleen and blood of Cl13-infected mice was marginally lower than that in Arm-infected animals, but was not reduced to a statistically significant extent. Again, type 1 IFN responses in LCMV Cl13-infection peaked before the peak in viral replication, although the viral titres in these mice rapidly reached higher titres than recorded in mice infected with LCMV Arm. It was unclear whether the increased viral replication

observed in LCMV Cl13-infected mice was assisted by the slight reduction in the magnitude of the type 1 IFN response (although it should be noted that persistent strains of LCMV are typically more resistant to the effects of type 1 IFN), or whether increased viral titres were in fact responsible for the reduction in the levels of type 1 IFN, which would indicate an LCMV-mediated impairment of host type 1 IFN production. Such an impairment could in turn impact upon the quality of the antiviral state induced in the host.

Apart from upregulating ISGs involved in establishing an antiviral state in the host during the early stages of infection, type 1 IFNs are also responsible for the maturation of cytotoxic T-cells needed for viral clearance. Resolution of LCMV infection requires production of IFN-γ cytokine and/or perforin-mediated killing of infected host cells. It is possible that the early shutdown of type 1 IFN production may lead to suboptimal priming of the CD8 T-cells resulting in a T-cell response which is able to contain and clear LCMV Arm infection but may not be sufficient to control the faster-replicating LCMV Cl13 strain. The reduction of circulating type 1 IFN in a productive, persistent infection has been reported to occur in other viral infections, including HIV, HCV and HBV, where it may be a factor promoting persistence of the infection (Anthony et al. 2004; Duan et al. 2004; Murakami et al. 2004; Siegal 2003).

Viral titres in the spleens of mice infected with LCMV Arm fell beyond the limit of detection by 3 weeks post-infection, as the acute infection was resolved. Following viral clearance, IFN-β and IFN-α mRNA transcripts in the spleen declined to baseline levels. By contrast, in the spleens of mice infected with LCMV Cl13, viral titres remained elevated at 3 weeks post-infection, but only low levels of type 1 IFN continued to be produced, (detected at the mRNA level). Confocal studies also confirmed this observation, as small numbers of IFN-α producing cells were

visualized in sections of LCMV Cl13-infected spleens. Previous studies, mainly carried out in mice infected at birth with LCMV also reported conflicting results: low but nonetheless elevated levels of IFN activity were reported in the plasma by some groups (Bukowski et al. 1983; Saron et al. 1982) whilst other reports did not find elevated levels of IFN in these animals (Holtermann, 1970; Wagner and Snyder, 1962; Traub and Kesting, 1963). The differing results of these studies possibly reflect the intermittent but low responses I observed in my experiments.

The identity of the cells which secrete type 1 IFN during infection with LCMV is still unclear. In acute LCMV Arm infection, it was reported that in contrast to other viral infections, plasmacytoid DCs are not the major producers of systemic type 1 IFN (Dalod et al. 2003). Depletion of splenic DCs with the DC antibody GR-1 did not alter the serum levels of type 1 IFN produced in response to LCMV. However, several labs have reported that GR-1 antibody does not completely deplete DCs *in vivo* (Asselin-Paturel et al. 2003; Belz et al. 2005). Studies conducted in our lab have found that acute infection with LCMV Arm does induce upregulation of type 1 IFN mRNA in $CD11c^+$ $B220^+$ plasmacytoid DCs, and also (to a much lower extent) in the $CD11c^+$ $B220^-$ conventional DC subset (Montoya et al. 2005).

My analysis of IFN-α/β mRNA levels in purified plasmacytoid and conventional DC subsets conducted on LCMV Arm or LCMV Cl13-infected mice confirmed the latter observations. Infection with LCMV Arm rapidly induced IFN-α/β production in splenic DCs, with plasmacytoid DCs upregulating IFN-α/β expression to a much higher extent than conventional DCs. Expression of type 1 IFN in plasmacytoid DCs also peaked earlier than in the conventional DC subset. Similar responses were recorded in plasmacytoid and conventional DC subsets isolated from the spleens of mice infected with LCMV Cl13. This was confirmed by immunofluorescent staining

of spleen sections taken shortly after infection from LCMV Arm and LCMV Cl13-infected mice, which showed some IFN-α production in plasmacytoid DCs. However, many of the IFN-α-secreting cells within the marginal zone area did not appear to be plasmacytoid DCs and it was not clear what the other IFN-α producing cells were. NK cells and macrophages as well as conventional DCs have been shown to upregulate type 1 IFN during viral infections, and repeating immunofluorescent staining of LCMV-infected spleen sections using mAbs specific for these cell types may be one way to identify the nature of these IFN-α producing cells. Alternatively, intracellular staining assays could be performed on splenocytes from infected mice, co-staining the cells with mAbs against IFN-α and markers for different lymphocyte populations, and the IFN-α producing cell type identified by FACs analysis.

Nonetheless, it is apparent that plasmacytoid DCs and to a lesser extent conventional DCs do contribute to the rising levels of type 1 IFN during the early phase of persistent LCMV infection. While the extent of upregulation of IFN-α/β in conventional DCs may not be equivalent to upregulation in plasmacytoid DCs on a per-cell basis, there are 10x more conventional DCs in the spleen, so as a total population, these cells probably make some contribution to the level of type 1 IFN in the system. Experiments that measure the type 1 IFN levels in LCMV-infected mice depleted of splenic DC subsets using other newly isolated plasmacytoid DC-specific antibodies such as mpDCA-1 may help resolve the uncertainty surrounding the relative contributions of plasmacytoid DCs, conventional DCs and other cell types to production of serum type 1 IFNs in LCMV-infected mice.

As previously observed in the spleen and the serum, type 1 IFN expression in plasmacytoid and conventional DCs isolated from LCMV Cl13-infected mice was low or undetectable during the later stages of infection. Immunofluorescent staining

of spleen sections from mice infected with LCMV Cl13 for 4 weeks showed widespread LCMV infection in the spleen but only low IFN-α production. IFN was produced by uninfected and a minority of infected cells, including principally plasmacytoid DCs (Figure 4.12). Importantly, there was little evidence of IFN-α production by infected cells other than plasmacytoid DCs. It thus appeared that the majority of infected splenocytes are unresponsive to the presence of LCMV viral components which would normally promote induction of type 1 IFNs. One explanation for this observation could be that infection with LCMV Cl13 may have mechanisms for impairing type 1 IFN production in the cells it infects.

The majority of cells, including fibroblast and conventional DC cells utilize the intracytoplasmic RIG-1, MDA5 and MAVS helicases to detect the presence of intracellular infection. By contrast plasmacytoid DCs possess multiple mechanisms for type 1 IFN induction: in addition to the RIG-I helicase intracytoplasmic pathway above, plasmacytoid DCs also use TLRs on the cell surface or in endosomes to sense extracellular or engulfed material (Haller et al. 2006; Kawai and Akira 2006). Conventional DCs also express TLR3 in the endocytic compartment which allows them to sense viral and other dsRNA molecules. The fact that the small number of infected cells in LCMV-infected mice that were observed to be producing type 1 IFN were plasmacytoid DCs suggests that LCMV may potentially impair type 1 IFN production via the intracellular helicase pathways used for detection of infection in the majority of infected cells, but may potentially leave plasmacytoid DC-specific type 1 IFN induction mechanisms intact.

Consistent with this, experiments in which BM-DCs and *in vitro* cell lines were infected with LCMV Arm or LCMV Cl13 revealed very poor induction of type 1 IFN. Fibroblast, BalbCl7 and DC cell lines, FSDC and D2SC/1, were infected as I wanted

to reflect the different cell types present *in vivo*. The DC cell lines were derived from spleens (D2SC/1)(Lutz et al. 1994) or dendritic cell precursors from fetal mouse skin (FSDC) (Girolomoni et al. 1995) and are reported to display DC phenotype that is more akin to conventional DCs than plasmacytoid DCs, as evidenced by stronger type 1 IFN responses to polyIC than R848 or CpG stimulation (Figure 5.14). D2SC/1 and FSDC have also been previously reported to upregulate detectable levels of type 1 IFN in response to an RNA virus, Sendai (Eloranta et al. 1997). It would have been of interest to conduct parallel experiments with a plasmacytoid-like cell line, but unfortunately no suitable cell lines were available.

A confounding factor in these experiments was that, unlike in *in vivo* tissue or primary cells where the background levels of type 1 IFN were non-existent or negligible (0-30U/ml), baseline levels of type 1 IFN in the supernatants of uninfected control cells fluctuated to varying degrees depending upon the cell line tested. Backgrounds in the supernatants of BalbC17 and DC cells fluctuated wildly from 0 to 125U/ml. Efforts to minimize the fluctuations, including culturing cells in low endotoxin serum, addition of polymyxin B to the culture medium, or making sure the cultures were not overgrown, did not overcome the fluctuation. This phenomenon has been previously described and is believed to be due to contact or growth-mediated stimulation (Hannigan and Williams 1986). While an upregulation of type 1 IFN was recorded immediately after LCMV infection in all the cell lines, it was difficult to be certain that the upregulation was virus-specific. The hallmark of type 1 IFN expression is a strong upregulation in response to viral infection, which was indeed observed when the same cells were infected with HSV or Sendai, where the maximal levels of type 1 IFN in the supernatant were clearly above the levels of the fluctuating background. However LCMV infection stimulated production of type 1 IFN activity

which was about 2-4-fold less than the other viral infections and barely above the background.

All in all, LCMV was able to infect all the cell lines tested, releasing infectious virions into the supernatant and generated a persistent infection with production of LCMV viral proteins in cells during the course of the persistent infection. Nonetheless, the type 1 IFN response to the infection was of a lower magnitude than the response seen in other viral infections. In agreement with *in vivo* observations, type 1 IFN production was not sustained beyond the first 3 days of infection even though virus continued to be produced at high titres. The presence of the high viral load did not induce any additional bursts of type 1 IFN production. While it may be possible that no further type 1 IFN was produced because viral components in the cell were sequestered away from the innate-sensing receptors present in the cytoplasm (e.g. RIG-I), or on the external cell surface or in intracellular organelles (e.g. TLRs), these results in *in vitro* systems also suggest the likelihood that there may be some impairment in the ability of infected host cells to recognize viral components and respond with production of the antiviral cytokine type 1 IFN.

As outlined above, most cells in the body detect the presence of an intracellular viral infection via the RIG-I helicase pathway that senses viral dsRNA (Haller et al. 2006; Kato et al. 2005), activating the TBK1/IKKe kinase complex to phosphorylate IRF-3 (Mori et al. 2004; Paz et al. 2006), enabling nuclear translocation of phosphorylated IRF3 and subsequent activation of IFN-β and IFN-α4 mRNA transcription (which starts off autocrine amplification of IFN-α/β cytokines and other interferon stimulated antiviral genes (as shown in Figure 6.1). Any block in this pathway would downregulate type 1 IFN production in these cells. However, a similar impairment would not completely block IFN-α/β induction in infected plasmacytoid DCs, as these

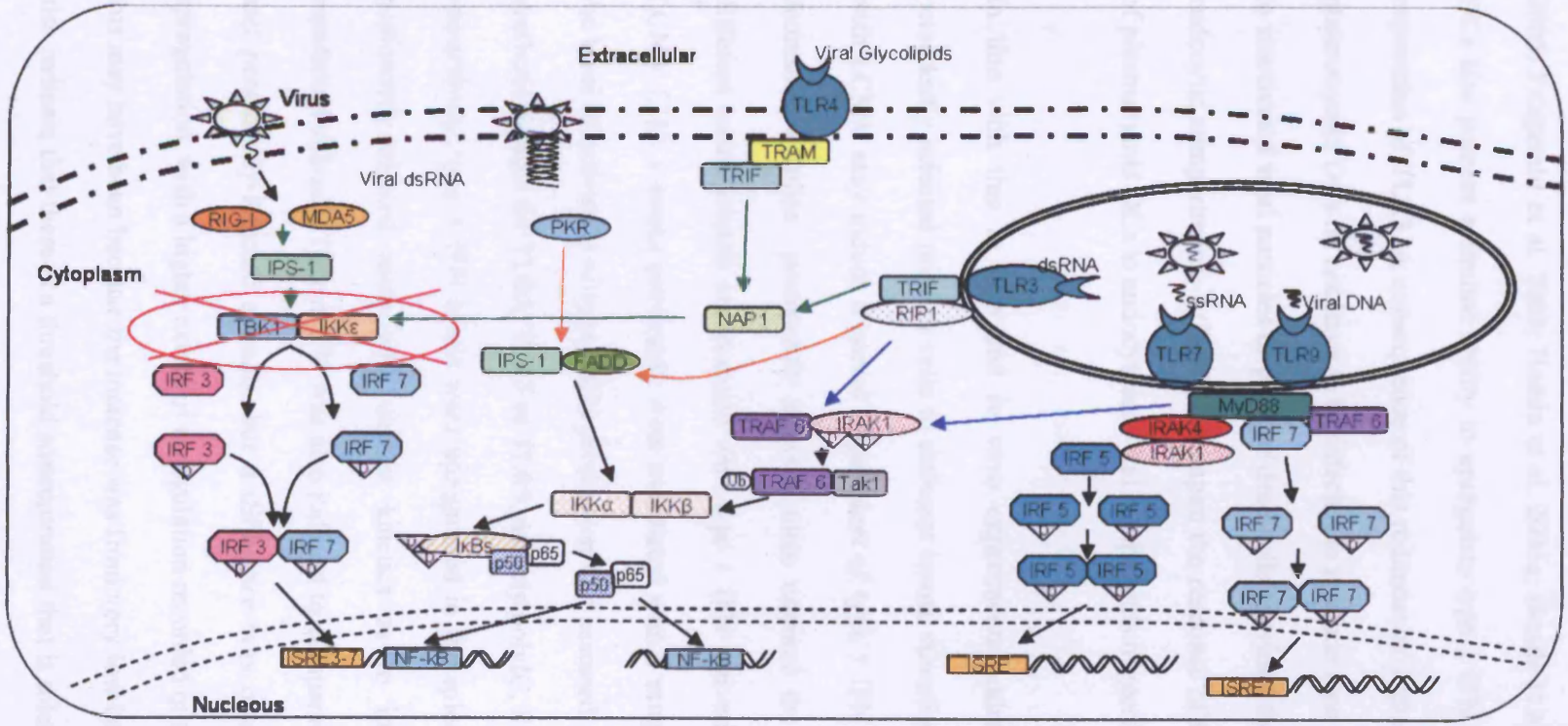

Figure 6.1 Pathways that may be involved in the transcriptional activation of type 1 IFN gene expression following virus infection

Overview of the signalling pathways leading to type 1 IFN production so far described are explained. The putative block along the RIG-I signalling pathway is indicated. Abbreviations are explained in the text (adapted from Kawai and Akira, 2006).

cells are also able to upregulate type 1 IFN expression via the TLR pathway, which relies on the activation of IRF7 and NF-κβ for type 1 IFN induction (Akira et al. 2006; Fitzgerald et al. 2003; Honda et al. 2005a; Honda et al. 2005b). Conventional DCs also possess a limited ability to upregulate type 1 IFN in this manner through expression of TLR3. A consequence of this redundancy is that conventional DCs and plasmacytoid DCs do not have to be infected to produce type 1 IFNs but can respond to inactivated viral particles or parts of dead cells provided they are taken up into the endocytic compartment. LCMV may impair the response of the cells to the presence of plasmacytoid DCs to endocytosed viral components triggered through TLRs.

In line with this *in vivo* and *in vitro* experiments addressing the response to persistently infected mice or cells to different innate stimuli suggested that infection with LCMV may induce a partial impairment of type 1 IFN induction. In order to determine whether persistently infected mice retained the capacity to recognize different innate stimuli and respond with type 1 IFN activation, mice infected with LCMV Cl13 3 weeks previously were inoculated with a range of innate stimuli and the level of activation of type 1 IFN production was assessed. Upon stimulation with synthetic ligands for TLR3, TLR7 or TLR9, namely polyIC, R848 or CpG 1826 DNA respectively, type 1 IFN levels were upregulated in the spleen and in the serum of persistently-infected mice with similar kinetics as to induction in stimulated uninfected animals. Type 1 IFN was also induced to comparable levels in uninfected and persistently-infected animals, but a difference was observed in the extent of upregulation, with a higher extent of upregulation recorded in the uninfected mice, but this may have been because the increase was from very low baseline levels. This may also indicate that there is a threshold concentration that is tolerated by the host before

a downregulation or expression plateau is enforced, perhaps as a tactic to avoid the toxic side effects of excessive levels of type 1 IFN (Pfau et al. 1983; Riviere et al. 1980).

The only marked difference in the response of uninfected and LCMV infected mice occurred when influenza PR8 was used as a stimulus. Influenza PR8, a live virus, was delivered i.p. so as to reduce the potential for complication of interpretation of results by differences in replication/spread of infection in uninfected and persistently-infected mice. *In vivo*, influenza replication is limited to epithelial cells of the upper and lower respiratory tract which express the protease necessary for viral activation (Rott et al. 1995). Therefore, administration of live influenza virus into the intraperitoneal cavity will result in attachment and entry of the virus into peritoneal cells, followed by replication but no release of new progeny virus. Infection with influenza virus has been reported to induce serum type 1 IFN production (Wabuke-Bunoti et al. 1986). Type 1 IFN may be produced, possibly in a RIG-I/MDA5-dependent manner, by cells responding to virus entry and replication, or may be produced by uninfected cells such as plasmacytoid DCs or other cell types responding to components of the virus such as ss or ds viral RNA, HA or other viral glycoproteins. Notably, influenza NS1 has been shown in *in vitro* studies to reduce type 1 IFN production and PKR activity in influenza-infected cells by sequestering dsRNA synthesized during viral replication. However, type 1 IFN production has still been observed in influenza-infected BM-DCs and human and mouse plasmacytoid DCs, where TLR7 is activated by viral ssRNA (Diebold et al. 2004; Krug et al. 2004; Lund et al. 2004).

When persistently infected mice were injected i.p with influenza virus, IFN-α and IFN-β mRNA in the spleen were upregulated as was the level of type 1 IFN activity in

the serum. The response was detectable as early as 6 hours post-stimulation and was sustained for at least 24 hours post-stimulation. However, the level of type 1 IFN activity induced in the serum of the mice was lower than the activity induced in uninfected mice administered with influenza in parallel. Further, mRNA levels of IFN-β, -α4 and other α subtypes were slightly lower in LCMV Cl13-infected mice and the IFN-stimulated gene Mx was upregulated to a much lower extent than in influenza-treated uninfected controls.

There may be several explanations for the reduced levels of type 1 IFN induced in persistently infected mice following inoculation with influenza virus. Firstly, the presence of LCMV Cl13 virus in the persistently-infected mice may have generated an ongoing antiviral state in the animals, making them more resistant to subsequent infection by influenza. Indeed, the spleens at 6, 12 and 24 hours post-stimulation did contain less influenza mRNA transcript than uninfected stimulated mice (Figure 4.15) when measured by Taqman quantitative RT-PCR. There may not thus have been as high level of viral components present to stimulate the cells, leading to a weaker systemic type 1 IFN response. Another cause of low type 1 IFN production may stem from the reduction in splenic DC numbers in the persistently-infected mice, because plasmacytoid dendritic cells represent a source of systemic type 1 IFN during influenza infections (Bruno et al. 2001; Diebold et al. 2004; O'Keeffe et al. 2002). In support of this, confocal images of influenza-stimulated spleen sections in uninfected mice showed a high frequency of co-localisation between IFN-α and mPDCA-1^+ cells (yellow cells) whilst a lower frequency of similar co-localisation was observed in the spleens of persistently-infected mice.

A third alternative explanation for the lower response is that the pathways responsible for type 1 IFN induction in response to influenza may be impaired in mice persistently

infected with LCMV Cl13. As reviewed above, these may include TLR-dependent pathways or intracellular helicase-dependent pathways. Stimulation of uninfected and persistently-infected mice with TLR ligands induced good type 1 IFN responses, so it might have been expected that a good response would also be induced to TLR ligands generated during influenza infection. However, it could be that prolonged exposure to high levels of LCMV viral components may have tolerised pathogen-responsive cells to further stimulation. The response observed following inoculation of synthetic TLR ligands could be a result of the high dose of ligand that was administered. By contrast, the levels of influenza PR8 was injected i.p. was low and the levels of stimuli generated during influenza infection may be lower than the levels of TLR ligands administered. Thus, it remains a possibility that cells in LCMV-infected mice may be tolerised to low doses of stimuli, leading the response to ligands in influenza PR8 to be impaired. In addition, the intracytoplasmic helicase pathway may be impaired in LCMV-infected cells, reducing the capacity of infected animals to respond to influenza infection via this mechanism.

Furthermore, it was found that *ex vivo* $CD11c^+$ DCs isolated from persistently-infected mice, which would consist of plasmacytoid and conventional DCs, still retained the capacity to produce type 1 IFN in response to high doses of exogenously added synthetic TLR ligands in culture, which again suggests that activation of type 1 IFN production via TLR 3, TLR7 and TLR9 pathways was functional (although it would have been of interest to examine the response to lower doses of TLR ligands too. However, this observation also suggests that should there be any impairment in the response to viral components *in vivo* and *in vitro* it would likely be in the intracellular sensing pathway.

Experiments on LCMV-infected DC and fibroblast cell lines also lend weight to this theory of infection associate impairment of IFN production through selected pathways. In a population of homogenous cells with either a DC (D2SC/1 and FSDC) or fibroblast (BalbCl7) phenotype, where >95% of cells were infected with LCMV, productive infection with another RNA virus, Sendai, which is dependent upon the RIG-I helicase pathway to upregulate type 1 IFN resulted in induction of reduced levels of IFN-α/β (Melchjorsen et al. 2005). In contrast, infection with HSV resulted in induction with HSV in induction of similar levels of type 1 IFN to those induced in uninfected cells (indicating the TLR 9 (Krug et al. 2004) and/or DNA sensing intracytoplasmic pathways (Stetson and Medzhitov 2006) are intact), and stimulation of LCMV Cl13-infected D2SC/1 and FSDC cells with synthetic TLR ligands also resulted in induction of an equivalent response to that observed in uninfected cells, strengthening the hypothesis that only the intracellular dsRNA-sensing pathway may be impaired.

On the basis of the data from *in vivo* and *in vitro* experiments, it is thus plausible that LCMV may be inhibiting the intracytoplasmic helicase pathway. Helicases such as RIG-I are responsible for production of type 1 IFNs by infected cells in response to RNA viruses, so it is not unexpected that if viral component(s) mediate impairment of IFN production in infected cells they would target the RIG-I or related induction pathway(s). Inactivation of the helicase pathway in LCMV-infected cells would offer a logical solution to all the observations discussed so far. Firstly, as depicted in Figure 6.1 (which depicts the same type 1 IFN induction pathways as outlined in Figure 1.1), impairment of RIG-I, IPS-1 or TBK1/IKKε would leave other arms of the type 1 IFN induction pathway (IRF-3, TRAF6, NFκβ etc) intact and ready for use by the TLR3/7/9 system, offering an explanation as to why I saw type 1 IFN produced when

infected mice/cells were exposed to TLR ligands (or HSV which is known to signal via TLR9).

The increase observed in serum levels of other inflammatory cytokines following inoculation of persistently-infected mice with TLR ligands can also be explained on the basis of selective impairment of activation of the innate response pathway. TNF-α and IL-6 can be upregulated by the presence of intracellular viral RNA activating TLR3/TLR7 and the adaptors TRAF6, NFκβ and IRF5 (Figure 6.1) (Kawai and Akira 2006). Increases in the levels of TNF-α and IL-6 upon stimulation with TLR ligands would be anticipated if NFκβ and TRAF6 are not affected by LCMV infection.

Is has also been proposed that an LCMV infection may dysregulate the JAK/STAT pathway by activating STAT2 and encouraging formation of STAT2:STAT2 homodimers not only in DCs but in all infected cells (Hahm et al. 2005). While the actual mechanism has not been clarified, if true, this would have the effect of disrupting the JAK/STAT pathway e.g. by reducing the amount of free STAT2 available to create STAT1:STAT2 heterodimers. Inhibitors of JAK/STAT not only suppress the production of anti-viral proteins but also the expression of RIG-I, MDA5, IPS-1, MAVS, IRF-3 and IRF-7 as they are all IFN-inducible proteins, and as a consequence, production of the 'second wave' IFNs is reduced, acting against the IFN-activating effect of viral dsRNA molecules which progressively accumulate in the cytoplasm (Haller et al. 2006). It is interesting to note that while the type 1 IFN mRNA levels were upregulated in a similar manner in the spleens of uninfected and persistently-infected mice after stimulation with synthetic TLR stimuli, Mx mRNA levels were consistently lower in stimulated persistently-infected spleens. Mx

expression is regulated by type 1 IFNs through activation of the JAK-STAT pathway. Low upregulation of Mx may reflect a defect in this pathway.

I investigated the possibility that components of LCMV may be impairing the type 1 IFN response. Viral proteins of LCMV have been implicated in impairment of other cellular functions, e.g. the NP protein interferes with the growth hormone transactivator factor GHF1 (Pit1) to dysregulate transcription of growth hormone (de la Torre and Oldstone 1992). The Z protein was reported to interact with certain host cell translation factors (Campbell Dwyer et al. 2000) and also pro-myelocytic protein (PML) – an oncoprotein induced by and involved in type 1 IFN responses. Binding of the Z protein to PML leads to redistribution of PML to the cytoplasm and inhibition of apoptosis; deregulation of host cell apoptotic mechanisms may help the virus to establish persistence (Chelbi-Alix et al. 1998; Lavau et al. 1995). Additionally, the Z protein which is reported to interact with the viral RNA polymerase, contains a zinc finger DNA binding motif which may interfere with the activity of other nucleic-acid binding proteins such as helicases or transcription factors.

Investigations were conducted in *in vitro* cell cultures. Plasmids containing individual proteins of LCMV, namely GP, NP or Z were stably transfected into DC cell lines as initial attempts to conduct experiments in transiently-transfected cells were not successful due to non-specific upregulation of type 1 IFN. I was not able to obtain a plasmid containing the L polymerase protein and so it was not tested. However, when these stably-transfected cells were infected with Sendai or HSV, there was no difference between the levels of type 1 IFN produced in the cells expressing GP, NP or Z and the levels of type 1 IFN induced in cells expressing blasticidin only or which were not transfected, suggesting that none of the proteins impaired type 1 IFN induction. Nonetheless, this result does not conclusively prove that the LCMV virus

or the viral proteins of LCMV does not mediate impairment of type 1 IFN induction. There may be several reasons why LCMV-induced IFN inhibition may not have been reproduced in the cell lines I generated expressing individual LCMV proteins: firstly, during a persistent viral infection, large concentrations of viral antigens accumulate in the infected cell, as confirmed by immunfluorescent staining of persistently infected cells (Figure 5.6). The stably-transfected cell lines did not express the transfected proteins at similar levels (see Figure 5.16), hence the concentration of the viral protein may not have been high enough for it to exert its inhibitory effects. In the case of the Z protein, I was not able to confirm expression of the protein as I lacked a specific anti-Z antibody. Alternative methods could have been employed to detect the presence of Z, perhaps by detecting the presence of Z mRNA by RT-PCR using Z-specific primers, but this would not give any information on the conformation, abundance and location of the Z protein in the stably transfected cells. Furthermore, although expression of GP and NP were confirmed by immunofluorescence, I was unable to determine the location/distribution of the proteins in the cell, which may have been altered through being expressed from an artificial plasmid construct.

Secondly, viral proteins may not be the inhibitory factor; if the intracellular pathway is blocked, it is likely that helicases are targeted and inhibition may be the result of an interation between helicases and viral RNA. Thirdly, the inhibitory effects may require the synergistic effects of a combination of viral proteins and possibly viral RNA e.g. it is hypothesized that the ligand for TLR7 is ssRNA complexed to nucleoprotein (Diebold et al. 2004). One possibility is that a similar ssRNA-viral protein complex expressed by LCMV could potentially attach to and block TLR7 activation. Indeed synthetic siRNAs of ~21-30 kb designed with 5' overhang structures on the ends efficiently block the activity of RIG-I (Marques et al. 2006).

Given more time, I would have liked to have been able to dissect the interactions between LCMV and components of the intracellular or type 1 IFN pathway. If the virus was downregulating components of the intracytoplasmic pathways, the levels of these factors in persistently infected cells could be analysed by FACS or quantitative real-time RT-PCR. Any evidence of *in situ* LCMV viral protein interaction with IRFs or helicases could be visualized by immunfluorescence while footprinting/Western blot assays could be conducted on persistently infected cells to determine if viral RNA interacts with helicases or TLRs. Alternatively, Western blot assays could be run to determine if there was interaction between viral proteins and STAT proteins and check whether any of the viral proteins interfered with STAT phosphorylation.

Infection of high dose LCMV in cell lines does not induce much type 1 IFN, presumably because in this situation, almost all the cells are infected and thus the entire population is impaired its ability to produce type 1 IFN via intracellular helicase pathways. GMCSF-matured BMDCs were found to produce some type 1 IFN in the first 24 hours after LCMV infection (Figure 5.2 & 5.3). These consist of cells with a conventional DC-like phenotype with TLR 2, 3, 4, 5, 6 and 9 expression (Datta et al. 2003; Lutz et al. 2000) in addition to the inracytoplasmic pathway (Melchjorsen et al. 2005). The type 1 IFN they produced may have been stimulated via TLR pathways. The DC cell lines D2SC/1 and FSDC also posses TLR3/7/9 expression as evidenced by their responsiveness to polyIC, R848 and CpG 1826 DNA (Figure 5.14); however, the modest levels of type 1 IFN activity induced by R848 in these cell lines indicate that they may express only low levels of TLR7. Impairment of the intracellular pathway by LCMV coupled with low TLR 7 expression on D2SC/1 and FSDC, may explain why very little type 1 IFN upregulation was observed in these cells following infection with LCMV.

In vivo, infection with acute and persistent strains/isolates of LCMV does stimulate, albeit for a short period, production of high levels of type 1 IFN. If LCMV impairs type 1 IFN production via helicase-dependent pathways in infected cells, it is likely that during the early or acute phase of infection, the bulk of the type 1 IFN produced is from uninfected cells such as plasmacytoid and conventional splenic DCs able to sense and respond the presence of viral components via PRRs such as TLRs, Although high levels of type 1 IFN are produced in the acute phase of LCMV infection, the response is rapidly downregulated; and there are only low levels of type 1 IFN production during the persistent phase of infection, as evidenced by analysis of spleen and serum levels of IFN-α/β mRNA and bioactivity in LCMV Cl13-infected mice at 3 weeks post-infection. There are a number of possible explanations for the downregulation of type 1 IFN production in early LCMV infection and the almost complete lack of type 1 IFN production in mice persistently infected with LCMV in spite of the systemic presence of infectious virus. As mentioned before, plasmacytoid and conventional DCs possess redundant pathways for type 1 IFN production, and so should be able to produce type 1 IFN even if they are infected. However, analysis of splenic DC subsets revealed that the number of conventional DCs, and subsequently plasmacytoid DCs is reduced following the acute phase of LCMV infection, and that during persistent LCMV infection the numbers of splenic $CD11c^+$ $B220^-$ and $CD11c^+$ mpDCA-1^+ or $B220^+$ DC cells were appreciably reduced. Confocal experiments using mpDCA-1 antibody confirmed the observation *in situ*, with a low frequency of pDCA-1^+ DCs being observed in spleen sections from LCMV Cl13-infected mice. The reduction in plasmacytoid DC numbers may be due in part to maturation into other DC subtypes, as as plasmacytoid DCs are able to become myeloid DCs upon

LCMV infection (Zuniga et al. 2004). However, there is also a dramatic overall reduction in splenic DC numbers in mice persistently-infected with LCMV.

Montoya *et al* (Montoya et al. 2002) showed that the drop in conventional DC numbers during acute infection with LCMV Arm was due to apoptosis (evidenced by Annexin V staining) of activated conventional DCs. Depletion of matured DCs by apoptotic mechanisms, may be accompanied by a defect in replacement of DCs from bone marrow precursors, resulting in a permanent depression of splenic DC numbers during the persistent stage of infection. It has been previously reported that there are profound alterations in hematopoietic functions in LCMV-infected mice, including suppression of pluripotent stem cells, granulocyte-macrophage progenitors, prothymocytes and erythroid progenitors (Bro-Jorgensen 1978; Bro-Jorgensen and Knudtzon 1977) and depletion of bone marrow lineage B cells (Borrow et al. 2005). A variety of mechanisms have been proposed to contribute to the abnormalities in hematopoietic functions observed during acute and chronic LCMV infection, including LCMV infection of bone marrow precursors, and infection-associated production of cytokines including IFN-α/β and TNF (Binder et al. 1997; Binder et al. 1998). The reduction in splenic DC numbers may be a consequence of infection of the $CD11^+$ precursor cells in the spleen which would, during normal steady state conditions, mature into conventional DCs (Naik et al. 2006). Additionally, it has been speculated that continuous type 1 IFN production in response to persistent LCMV Cl13 infection suppresses the development of bone marrow precursor cells into DCs via a yet undefined STAT2-dependent pathway (Hahm et al. 2005).

In addition to the numbers of plasmacytoid and conventional DCs in the spleen of mice persistently-infected with LCMV being reduced, there is also very little type 1 IFN from the remaining cells as the levels of IFN-α/β mRNA in the few DCs present

in the spleen of persistently-infected mice were also low/undetectable. IFN-β, α-4 and non-α4 mRNA levels in plasmacytoid DCs and conventional DCs over the course of a persistent LCMV Cl13 infection demonstrated the same trend of early peak followed by a swift fall as was seen in the whole spleen (Figure 4.8). Plasmacytoid DCs appear to make a larger and more sustained contribution of type 1 IFN production during the early stages of infection than conventional DCs, although conventional DCs also upregulate type 1 IFN expression. There may be two reasons for this: first, plasmacytoid DCs are specialized type 1 IFN producing cells, second, plasmacytoid DCs, by virtue of TLR7 expression, can respond to ssRNA and LCMV, being a single-stranded RNA virus may be a stronger inducer of TLR7-mediated type 1 IFN production. By contrast, conventional DCs express TLR3 and are adapted to produce type 1 IFNs following endocytosis of dsRNA. Whilst dsRNA generated in infected cells during the LCMV replication process likely stimulates production of type 1 IFN by conventional DCs, this may accumulate with relatively delayed kinetics and possibly to lower levels than ss RNA.

Nonetheless, there is evidence that while these DCs may not respond to the presence of LCMV infection, they are able to produce type 1 IFN in response to synthetic TLR stimuli and also to live virus. *Ex vivo* $CD11c^+$ DCs from persistently-infected mice were still able to produce type 1 IFN when stimulated with polyIC, R848, or CpG (Figure 4.21) and IFN-α-producing infected plasmacytoid DCs were visible in spleen sections of infected mice after inoculation with R848, CpG or influenza PR8 (Figure 4.20). The question then arises as to why these cells were not producing type 1 IFN *in vivo* in response to the presence of high viral components, which could have also activated similar TLR. One possibility, as mentioned earlier, is that the continual presence of viral components may have tolerised the infected cells to a certain level of

innate stimuli, perhaps by downregulating expression of their innate sensing PRRs, as a measure to limit type 1 IFN-mediated immunopathology. Thus these cells may only be able to respond to high doses of a stimulus, such as those I used in my experiments: the levels of polyIC, R848 or CpG 1826 DNA added into the culture of *ex vivo* $CD11c^+$ DCs may be many times higher than physiological levels (Figure 4.18). By comparison, the levels of viral components available in the host to trigger TLRs may be relatively low. Alternatively, it is also possible that while high levels of infectious virus are present in LCMV-infected mice, the viral components that would activate TLRs, such as LCMV nucleic acid (ds and ssRNA) may be largely sequestered in infected cells, and not available to bind to TLRs.

I also analysed the activation status DCs from persistently-infected mice in order determine whether downregulation of type 1 IFN induction was accompanied by other changes in the activation state of these infected DCs. LCMV Cl13 is known to infect $CD11c^+$ DCs (Salvato et al. 1991; Sevilla et al. 2000). Previous studies have suggested that LCMV Cl13 infection of adult wild-type C57BL/6 mice prevented DC maturation, as the DCs exhibited reduced expression of co-stimulatory molecules, leading to immunosuppression of the host and ineffective clearance of the virus (Sevilla et al. 2003; Sevilla et al. 2004). However, in these studies, DCs subsets from persistently-infected mice were defined on the basis of $CD11c^+$ and $CD8\alpha^+$ expression from a preparation of whole spleen splenocytes treated with EDTA to disrupt T-cell-DC complexes. While CD11c expression is routinely used as a marker for splenic dendritic cell populations, it is a β2 intergrin with critical importance for the development of functional immune responses in its own right. NK cells are also known to be $CD11c^+$ while CD11c expression on T cells has been detected in a population of intestinal intraepithelial lymphocytes (Huleatt and Lefrancois 1995) and

crucially, on activated LCMV-specific $CD8^+$ T cells following systemic LCMV infection (Lin et al. 2003). The validity of the conclusions of the studies carried out by Sevilla *et al* is thus questionable as it was likely that the reduced expression of co-stimulatory markers observed may be the result of a dilution effect stemming from observations made of a DC population that was contaminated with $CD11c^+$ $CD8\alpha^+$ activated T-cells (which do not express co-stimulatory molecules).

Initial FACS analysis conducted on pooled lymphocytes from mice infected 3 weeks previously with LCMV Cl13 which were enriched on a Nycodenz column and positively selected for CD11c expression confirmed the presence of a large proportion of cells that were $CD11c^+$ but expressing non-DC markers in the preparation. A large proportion of contaminating cells appeared to be T-cells, as expected. Some of the contaminants were B-cells, which are another population reported to express low levels of CD11c, but is increased on the surface of B cells from B-cell chronic lymphocytic leukemia (B-CLL) sufferers (D'Arena et al. 2001). While DCs demonstrate plasticity and have been reported to upregulate other markers, including F4/80 (Ruedl et al. 1996) following activation, no reports could be found of DCs upregulating CD3, CD19 or DX5. A negative selection cocktail consisting of CD3, CD19, DX5 and F4/80 was thus employed to remove $CD11c^+$ non-DC cells. The antibodies were biotinylated and streptavidin-conjugated Dynal beads were subsequently used to remove the contaminating cells. Using this approach, I found that after depletion, there were still $CD11c^+$ cells that expressed CD3, CD19 or DX5. A small proportion of these cell were also pDCA-1 or CD4 positive, although many more $CD11c^+$ $CD8^+$ cells (most likely activated T-cells) were present (Figure 4.7). As such, only a minority of the $CD11c^+$ non-DC cells remaining in the prep would be

included in the gated $CD11c^+$ $pDCA\text{-}1^+$ or $CD11c^+$ $CD4^+$ populations, but the majority of the cells in the $CD11c^+$ $CD8\alpha^+$ population would likely be non-DCs.

Levels of co-stimulatory markers on purified $CD4^+$ and $mpDCA\text{-}1^+$ DC subsets were found to be enhanced over levels in uninfected DCs (Figure 4.8), consistent with a mature phenotype. Levels of ICAM-1, CD40, CD80, CD86 and MHC Class I were upregulated in $pDCA\text{-}1^+$ DCs and $CD4^+$ DC subsets of mice at 3 weeks post-infection. In agreement with previous reports (Martin et al. 2002; Montoya et al. 2005), the $pDCA\text{-}1^+$ subset expressed lower levels of co-stimulatory molecules than conventional DC subsets, consistent with these cells playing a more minor role as a APCs (as they are believed to be predominantly specialized type 1 IFN producers). Therefore, there does not appear to be a defect in 2 of the DC subsets studied. The results are in line with observations of Homann et al (2004) who studied the activation status of DCs in mice that were persistently-infected at birth with LCMV and found the DCs in these mice to be activated (Homann et al. 2004). These observations suggest that persistent LCMV infection, regardless of the viral strain involved, and whether infection is established by T-cell exhaustion in adult mice or tolerance at birth, is associated with activation of $pDCA\text{-}1^+$ and $CD4^+$ DCs.

Surface expression of co-stimulatory molecules on the $CD8\alpha^+$ DC subset, apart from CD86, appear to be reduced as assessed by mean fluorescent intensity (MFI); however, this may not reflect the true MFI levels of $CD8\alpha^+$ DCs as it is probable that the population contained a high level of non-DC $CD8\alpha^+$ cells, most likely T-cells, which would have reduced the overall per-cell MFI levels. Thus persistently-infected $CD8\alpha^+$ DC may be expressing higher levels of co-stimulatory molecules than measured here. Future experiments employing 6-colour FACS analysis to allow staining with a larger panel of phenotypic markers, e.g CD3 staining in conjunction

with CD8α marker to exclude T-cells in order to definitively isolate the CD8α DC subset, should resolve this uncertainty.

It is unclear what the consequences of chronic DC activation in LCMV-infected mice may be. The presence of activated DCs may help to drive exhaustion of LCMV-specific CD8 cytotoxic T cells, promoting viral persistence. Activated DCs an also be tolerogenic; e.g. DC matured in the presence of TNF which are able to stimulate CD8 T-cell proliferation, but these cells are swiftly deleted leading ultimately to tolerance (Albert et al. 2001). Wilson *et al* reported that when DCs were preactivated with CpG, LPS or poly IC and then infected with HSV, no HSV specficic T-cell proliferation was recorded as the DCs were not able to present HSV (Wilson et al. 2006) likewise the continual presence of LCMV viral components may be mimicking the effects of pre-treatment with TLR ligands, and may impair DC presentation of LCMV antigens to T cells.

Experiments investigating the primary response as well as the generation and maintenance of memory responses in these persistently-infected mice to unrelated protein antigens (e.g. CGG/OVA) delivered with or without different adjuvants (e.g. using tetramer staining to follow T cell responses and in vivo CTL or ELISPOT assays to measure T-cell functions) may help provide answers as to the true activation status and functional capacity of the DCs in mice persistently-infected with LCMV.

In addition, had I more time, it would have been interesting to determine the activation status of other innate cells such as NK cells during persistent infection comparing their activity to that of cells from uninfected and acutely-infected animals. IFN-γ expression was barely detectable in the serum of persistently-infected mice. This may be a reflection of the non-functional LCMV-specific CTL immunity, but may also suggest that NK cell functions are not chronically activated in mice

persistently-infected with LCMV, as NK cells represent another important source of IFN-γ. However, previous studies have shown that NK cells in mice persistently-infected with LCMV at birth are more activated than NK cells in uninfected mice (Bukowski et al. 1983).

Yet another explanation for the downregulation of type 1 IFN production in persistently infected animals may be that it is a host-mediated mechanism to prevent the immunopathological effects stemming from high levels of systemic type 1 IFN. The presence of excessive levels of type 1 IFN for prolonged periods may impact on both the immune system as well as general functions, as described in the introduction. After an initial first and second wave of upregulation and amplification of type 1 IFN and IFN-stimulated anti-viral cytokines, a third wave of gene transcription is activated that produces inhibitors of type 1 IFN. It may be possible that persistent stimulation induces production of these inhibitors.

In summary, during the acute phase of an LCMV infection, type 1 IFN is probably mainly induced through the TLR pathway (with cell types including plasmacytoid DCs and conventional DCs detecting ssRNA through TLR7 or the viral GP through an unidentified PRR). It is unlikely that high levels of the type 1 IFN are produced by infected cells through the helicase pathway, as data from this study suggests this pathway may be impaired. The type 1 IFN produced induces an antiviral state in uninfected cells, upregulates antiviral proteins and also activates the immune system to control viral spread. However, type 1 IFN-resistant strains/isolates of LCMV such as LCMV Cl13 are able to combat these measures, and are thus able to spread at a faster rate, outstripping control by the host T cell response. As infection progresses, the type 1 IFN response is downregulated, perhaps in part as a result of viral impairment and possibly also due to DC "tolerance" and DC loss, and also that homeostatic

mechanisms that limit type 1 IFN production and associated immunopathological damage. The activation and subsequent loss and/or tolerance of DCs may have effects on the host's CTL response which is first stimulated and then becomes exhausted in the presence of high viral titres (possibly through a PD-1 dependent mechanism (Barber et al. 2006; Fuller and Zajac 2003; Moskophidis et al. 1993; Zajac et al. 1998), enabling the virus to persist in the host.

During persistent LCMV infection, little if any type 1 IFN is likely induced through the helicase pathway, but there is a low level of IFN-produced by cell types including plasmacytoid DCs, probably via TLR pathways. However a reduced number of DCs is present in the host is likely as a consequence of LCMV infection-associated activation and enhanced DC turnover associated with impaired replenishments of DCs from the bone marrow. The low numbers of splenic DCs remaining in mice persistently-infected with LCMV are nonetheless activated and express co-stimulatory molecules. There is some ongoing production of type 1 IFN by these cells, and in response to stimulation with TLR ligands, they are able to upregulate production of type 1 IFN. These cells may thus participate in defence against secondary infections; although the reduced numbers of these cells present, coupled with the impairment in type 1 IFN production through helicase-dependent pathways may result in defective control of other infections, in particular RNA virus infections.

At the time of preparation of this report, a paper was published which demonstrated that impairment of type 1 IFN production during LCMV infection was caused by the LCMV NP protein preventing translocation of IRF3 (Martinez-Sobrido et al. 2006). Using an *in vitro* system, the authors showed that type 1 IFN induction was blocked only at the level of induction, through impairment of IRF3 and not at the level of signaling (i.e. the JAK/STAT pathway). The results of this paper fit very well the

findings of this study, which predicted that an impairment was likely to occur along the RIG-I helicase pathway, which is dependent on the activation of IRF3 to induce transcription of IFN-α/β genes (Figure 6.1), unlike the TLR pathway which is able to utilize alternative transcription factors, namely IRF7 and NF-κB, to upregulate IFN-α/β expression.

As LCMV is a natural pathogen of mice, the model of persistent infection analysed here may provide a good reflection of what occurs in during other virus infections. The findings made in this study may thus contribute to increasing our current understanding of the state of the innate immune system during human persistent viral infections of clinical relevance.

The observation that splenic DC numbers are reduced during persistent LCMV infection is similar to observations made in some chronic human infections. During chronic HIV infection, the number of plasmacytoid DCs is reduced (Donaghy et al. 2004; Schmidt et al. 2006; Soumelis et al. 2001). This may be due in part to direct infection by the virus, but it also may be due to impaired haemopoietic mechanisms analogous to the situation in persistent LCMV infection. Like LCMV, HCV is able to block type 1 IFN production in the cells it infects, These observations I made in the LCMV model suggest that infected cells may still be able to produce type 1 IFN, if the correct type 1 IFN induction pathway is stimulated.

It is important to unravel the mechanisms by which type 1 IFN production is impaired in chronic viral infections because this information may help direct the design of more effective therapies. For example, if impairment occurs only at the IFN induction step, then administration of type 1 IFN may be helpful in stimulating cells to establish an antiviral state. Such therapies are already used to treat HCV and HBV infections, where it is known that the virus-induced impairment occurs at the level of type 1 IFN

induction (Bertoletti and Gehring 2006; Murakami et al. 2004; Tsubouchi et al. 2004). However, such therapies would not be useful in situations where the block occurs in the JAK/STAT pathway.

Studies like this also give insight into the consequences of impairment of type 1 IFN production for therapeutic vaccination. If for example, a chronic viral infection such as HCV, establishes a persistent infection by impairing the RIG-I pathway, then vaccination using a live viral vector may not be effective as live viruses would normally stimulate production of type 1 IFN (which has important adjuvant properties (Le Bon et al. 2003; Le Bon et al. 2001)), via the RIG-I pathway. The choice of adjuvants could be adjusted, e.g. use of a TLR ligand such polyIC or R848 in conjuction with the vaccine may be a better strategy than use of a live viral vector alone. Additionally, if an antiviral state is established in patients with a chronic viral infection, as was observed in chronic LCMV infection, a live viral vector, which relies on replication in the host for effective results, may not be the best strategy to use. The LCMV model of persistent infection could be used to test out these vaccine strategies for use in infections where the RNA helicase pathways are impaired.

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